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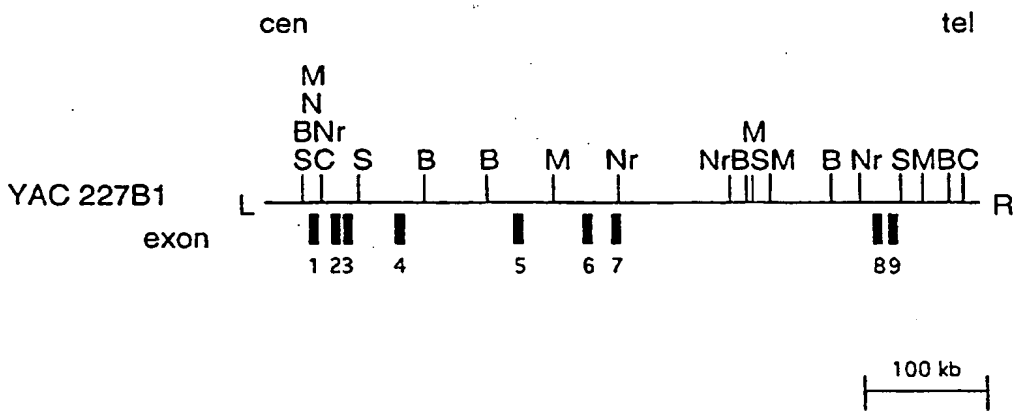
(54) Title: GENE SEQUENCE FOR SPINOCEREBELLAR ATAXIA TYPE 1 AND METHOD FOR DIAGNOSIS



(57) Abstract

The present invention provides an isolated DNA molecule of the autosomal dominant spinocerebellar ataxia type 1 gene, which is located within the short arm of chromosome 6. This isolated DNA molecule is preferably located within a 3.36 kb *Eco*RI fragment, i.e., an *Eco*RI fragment containing about 3360 base pairs, of the SCA1 gene. The isolated sequences contain a CAG repeat region. The number of CAG trinucleotide repeats (n) is ≤ 36, preferablyn = 19-36, for normal individuals. For an affected individual n > 36, preferably n ≥ 43.

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GENE SEQUENCE FOR SPINOCEREBELLAR ATAXIA TYPE 1 AND METHOD FOR DIAGNOSIS

Statement of Government Rights

The present invention was made with government support under Grant Nos. NS 22920 and 27699, awarded by the National Institutes of Health. The Government has certain rights in this invention.

Background of the Invention

The spinocerebellar ataxias are a heterogeneous group of degenerative neurological disorders with variable clinical features resulting from degeneration of the cerebellum, brain stem, and spinocerebellar tracts. The clinical symptoms include ataxia, dysarthria, ophthalmoparesis, and variable degrees of motor weakness. The symptoms usually begin during the third or fourth decade of life, however, juvenile onset has been identified. Typically, the disease worsens gradually, often resulting in complete disability and death 10-20 years after the onset of symptoms. Individuals with juvenile onset spinocerebellar ataxias, however, typically have more rapid progression of the phenotype than the late onset cases. A method for diagnosing spinocerebellar ataxias would provide a significant step toward its treatment.

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant disorder which is genetically linked to the short arm of chromosome 6 based on linkage to the human major histocompatibility complex (HLA). See, for example, H. Yakura et al., N. Engl. J. Med., 291, 154-155 (1974); and J.F. Jackson et al., N. Engl. J. Med., 296, 1138-1141 (1977). SCA1 has been shown to be tightly linked to the marker D6S89 on the short arm of chromosome 6, telomeric to HLA. See, for example, L.P.W. Ranum et al., Am. J. Hum. Genet., 49, 31-41 (1991); and H.Y. Zoghbi et al., Am. J. Hum. Genet., 49, 23-30 (1991). Recently, two families with dominantly inherited ataxia failed to show detectable linkage with HLA markers but were found to have SCA1 when studied for linkage to D6S89, demonstrating the superiority of the latter marker for study of ataxia families. See, for example, B.J.B. Keats et al., Am. J. Hum. Genet., 49, 972-977 (1991). The identification and cloning of the SCA1 gene could provide methods of detection that would be extremely valuable for both family counseling and planning medical treatment.

Summary of the Invention

The present invention is directed to a portion of an isolated 1.2-Mb region of DNA from the short arm of chromosome 6 containing a highly polymorphic CAG repeat region in the SCA1 gene. This CAG repeat region is unstable (i.e., highly variable within a population) and is expanded in individuals with the autosomal dominant neurodegenerative disorder spinocerebellar ataxia type 1 (i.e., affected individuals generally have more than 36 CAG repeats). Southern and PCR analyses of the CAG repeat region demonstrate correlation between the size of the expanded repeat region and the age-of-onset of the disorder (with larger alleles, i.e., more repeat units, occurring in juvenile cases), and severity of the disorder (with larger alleles, i.e., more repeat units, occurring in the more severe cases).

Specifically, the present invention provides a nucleic acid molecule containing a CAG repeat region of an isolated autosomal dominant spinocerebellar ataxia type 1 gene (herein referred to as "SCA1"), which is located within the short arm of chromosome 6. The SCA1 gene contains a region that encodes a protein herein referred to as "ataxin-1." The nucleic acid molecule of the present invention can be a single or a double-stranded polynucleotide. It can be genomic DNA, cDNA, or mRNA of any size as long as it includes the CAG repeat region of an isolated SCA1 gene. Preferably, the nucleic acid molecule includes the SCA1 coding region and is of about 2.4-11 kb in length. It can be the entire SCA1 gene (whether genomic DNA or a transcript thereof) or any fragment thereof that contains the CAG region of the gene. One such fragment is an *Eco*RI fragment of the SCA1 gene, i.e., a fragment obtained through digestion with *Eco*RI endonuclease restriction enzyme, containing about 3360 base pairs having therein a polymorphic CAG repeat region. By polymorphic CAG repeat region it is meant that there are repeating CAG trinucleotides in this portion of the gene that can vary in the number of CAG trinucleotides. The number of trinucleotide repeats can vary from as few as 19, for example, to as many as 81, for example, and larger.

For a normal individual, $n \leq 36$ in the $(CAG)_n$ region, i.e., n = 2-36, and typically n = 19-36. This region in a normal allele of the SCA1 gene is optionally interrupted with CAT trinucleotides. Typically, there are no more than about 3 CAT trinucleotides, either individually or in combination, within any

$(CAG)_n$ region. The $(CAG)_n$ region of this isolated sequence is unstable, i.e., highly variable within a population, and larger, i.e., expanded, in individuals who have symptoms of the disease, or who are likely to develop symptoms of the disease. For an affected individual, i.e., an individual with an affected allele of the SCA1 gene, $n > 36$ in the $(CAG)_n$ region, and typically $n \geq 43$. One isolated DNA molecule of the SCA1 gene is about 3360 base pairs in length as shown in Figure 1. The sequences of a portion of the *Eco*RI fragment within the SCA1 gene of several affected individuals is shown in Figure 2. The entire 10,660 nucleotides of the SCA1 gene transcript are shown in Figure 15 (the entire SCA1 gene spans about 450 kb of genomic DNA).

The present invention is also directed to isolated oligonucleotides, particularly primers for use in PCR techniques and probes for diagnosing the neurodegenerative disorder SCA1. The oligonucleotides have at least about 11 nucleotides and hybridize to a nucleic acid molecule containing a CAG repeat region of an isolated SCA1 gene. The hybridization can occur to any portion of a nucleic acid molecule containing a CAG repeat region of the SCA1 gene. Preferably, the oligonucleotides hybridize to a 3.36 kb *Eco*RI fragment of an SCA1 gene having a CAG repeat region. Alternatively stated, each oligonucleotide is substantially complementary (having greater than 65% homology) to a nucleotide sequence having a CAG repeat region, i.e., a $(CAG)_n$ region, preferably to a 3.36-kb *Eco*RI fragment of the SCA1 gene. If the oligonucleotide is a primer the molecule preferably contains at least about 16 nucleotides and no more than about 35 nucleotides. Furthermore, preferred primers are chosen such that they produce a primed product of about 70-350 base pairs, preferably about 100-300 base pairs. More preferably, the primers are chosen such that nucleotide sequence is complementary to a portion of a strand of an affected or a normal allele within about 150 nucleotides on either side of the $(CAG)_n$ region, including directly adjacent to the $(CAG)_n$ region. Most preferably, the primer is selected from the group consisting of CCGGAGCCCTGCTGAGGT (CAG-a), CCAGACGCCGGGACAC (CAG-b), AACTGGAAATGTGGACGTAC (Rep-1), CAACATGGGCAGTCTGAG (Rep-2), CCACCACTCCATCCCAGC (GCT-435), TGCTGGGCTGGTGGGGGG (GCT-214), CTCTCGGCTTTCTTGGTG (Pre-1), and GTACGTCCACATTTCCAGTT (Pre-2). These primers substantially correspond to those shown in Figure 3.

They can be used in any combination for sequencing or producing amplified nucleic acid molecules, e.g., DNA molecules, using various PCR techniques. Preferably, for amplification of the DNA molecule characteristic of the SCA1 disorder, Rep-1 and Rep-2 is the primer pair used. As used herein, the term "amplified DNA molecule" refers to DNA molecules that are copies of a portion of DNA and its complementary sequence. The copies correspond in nucleotide sequence to the original DNA sequence and its complementary sequence. The term "complement", as used herein, refers to a DNA sequence that is complementary (having greater than 65% homology) to a specified DNA sequence. The term "primer pair", as used herein, means a set of primers including a 5' upstream primer that hybridizes with the 5' end of the DNA molecule to be amplified and a 3' downstream primer that hybridizes with the complement of the 3' end of the molecule to be amplified.

Using the primers of the present invention, PCR technology can be used in the diagnosis of the neurological disorder SCA1 by detecting a region of greater than about 36 CAG repeating trinucleotides, preferably at least 43 repeating CAG trinucleotides. Generally, this involves treating separate complementary strands of the DNA molecule containing a region of repeating CAG codons with a molar excess of two oligonucleotide primers, extending the primers to form complementary primer extension products which act as templates for synthesizing the desired molecule containing the CAG repeating units, and detecting the molecule so amplified.

An oligonucleotide that can be used as a gene probe for identifying a nucleic acid molecule, e.g., a DNA molecule, containing a CAG repeat region of the SCA1 gene is also provided. The gene probe can be used for distinguishing between the normal and the larger affected alleles of the SCA1 gene. The gene probe can be a portion of a nucleotide sequence of the SCA1 gene itself (e.g., a 3.36-kb *Eco*RI fragment or portion thereof), complementary to it, or hybridizable to it or the complement. It is of a size suitable for forming a stable duplex, i.e., having at least about 11 nucleotides, preferably having at least about 15 nucleotides, more preferably having at least about 100 nucleotides (for effective Southern blotting), and most preferably having at least about 200 nucleotides. The probe can contain any portion of the $(CAG)_n$ region, although this is not a requirement. It is desirable, however, for the probe to contain a portion of the nucleic acid molecule on either

side of the $(CAG)_n$ region. There is generally no maximum size limitation for such probes. In fact, the entire SCA1 gene could be a probe.

The gene probe of the present invention is useable in a method of diagnosing a patient for SCA1. A particularly preferred method of diagnosis involves detecting the presence of a DNA molecule containing a CAG repeat region of the SCA1 gene. Specifically, the method includes the steps of digesting genomic DNA with a restriction endonuclease to obtain DNA fragments; preferably, separating the fragments by size using gel electrophoresis; probing said DNA fragments under hybridizing conditions with a detectably labeled gene probe that hybridizes to a nucleic acid molecule containing a CAG repeat region of an isolated SCA1 gene; detecting probe DNA which has hybridized to said DNA fragments; and analyzing the DNA fragments for a $(CAG)_n$ region characteristic of the normal or affected forms of the SCA1 gene.

The present invention also provides a protein (or portions thereof) encoded by the SCA1 gene and antibodies (polyclonal or monoclonal) produced from the protein or portions thereof. The antibodies can be used in methods of isolating antigenic protein expressed by the SCA1 gene. For example, they can be added to a biological sample containing the antigenic protein to form an antibody-antigen complex, which can be isolated from the sample and exposed to amino acid sequencing of the antigenic protein. This can be done while the protein is still complexed with the antibody.

Thus, the present invention provides methods to determine the presence or absence of an affected form of the SCA1 gene, which can be based on RNA- or DNA-based detection methods (preferably, the methods involve isolating and analyzing genomic DNA) or on protein-based detection methods. These methods include, for example, PCR-based methods, direct nucleic acid sequencing, measuring expression of the SCA1 gene by measuring the amount of mRNA expressed or by measuring the amount of ataxin-1 protein expressed. The methods of the present invention also include determining the size of the repeat region of the nucleic acid or amino acid molecules.

As used herein, the term "isolated (and purified)" means that the nucleic acid molecule, gene, or oligonucleotide is essentially free from the remainder of the human genome and associated cellular or other impurities. This does not mean that the product has to have been extracted from the human genome;

rather, the product could be a synthetic or cloned product for example. As used herein, the term "nucleic acid molecule" means any single or double-stranded RNA or DNA molecule, such as mRNA, cDNA, and genomic DNA.

As used herein, the term "SCA1 gene" means the deoxyribopolynucleotide located within the short arm of chromosome 6 between markers D6S89 and D6S274 of about 450 kb (10.5-11 kb transcript) containing an unstable CAG repeat region. This term, therefore, refers to numerous unique genes that are substantially the same except for the content of the CAG repeat region. A representative example of the SCA1 gene transcript for a normal individual is shown in Figure 15. Included within the scope of this term is any ribo- or deoxyribo-polynucleotide containing zero, one or more nucleotide substitutions that also encodes the protein ataxin-1. Included in the term "SCA1 gene" is any polynucleotide as described in the previous sentence that has different numbers of CAG and/or CAT repeats in the polymorphic CAG repeat region. It is understood also that the term "SCA1 gene" includes both the polypeptide-encoding region and the regions that encode the 5' and 3' untranslated segments of the mRNA for SCA1. Although the SCA1 gene described herein is described in terms of the human genome, it is envisioned that other mammals, e.g., mice, may also have a very similar gene containing a CAG repeat region that could be used to produce oligonucleotides, for example, that are useful in diagnosing the SCA1 disorder in humans.

As used herein, the term "ataxin-1" means the gene product of the SCA1 gene, i.e., protein encoded by the open reading frame of the SCA1 gene and any protein substantially equivalent thereto, including all proteins of different lengths (e.g., 20-90 kD, preferably 60-90 kD) encoded by said open reading frame which start at each in-frame ATG translation start site. The term "ataxin-1" further includes all proteins with essentially the same N-terminal and C-terminal sequences but different numbers of glutamine (Q) and/or histadine (H) repeats (primarily glutamine repeats) in the polymorphic repeat region.

As used herein, the term "polymorphic CAG repeat region" or simply "CAG repeat region" means that region of the SCA1 gene that encodes a string of polyglutamate residues that varies in number from individual allele to individual allele, and which can range in number from 2 to 80 or more. Moreover, the polymorphic CAG repeat regions can contain CAT (encoding histidine) in place of

CAG, although CAT is much less common than CAG in this region. It is to be understood that when referring to nucleic acid molecules containing the CAG repeat region, this includes RNA molecules containing the corresponding GUC repeat region.

As used herein, an "affected" gene refers to the allele of the SCA1 gene that, when present in an individual, is the cause of spinocerebellar ataxia type 1, and an "affected" individual has the symptoms of autosomal dominant spinocerebellar ataxia type 1. Individuals with only "normal" SCA1 genes, do not possess the symptoms of SCA1. The term "allele" means a genetic variation associated with a coding region; that is, an alternative form of the gene.

As used herein, "hybridizes" means that the oligonucleotide forms a noncovalent interaction with the stringency target nucleic acid molecule under standard conditions. The hybridizing oligonucleotide may contain nonhybridizing nucleotides that do not interfere with forming the noncovalent interaction, e.g., a restriction enzyme recognition site to facilitate cloning.

Brief Description of the Drawings

Figure 1. Sequence of the 3.36 kb *Eco*RI fragment of the normal SCA1 gene located within the short arm of chromosome 6. It is within this fragment that mutations occur in the CAG repeat region which are associated with autosomal dominant spinocerebellar ataxia type 1.

Figure 2. Sequence information for five affected individuals in the CAG repeat region, i.e., the CAG trinucleotide repeat, and its flanking regions of the SCA1 gene located within a short arm of chromosome 6.

Figure 3. Sequence of the CAG trinucleotide repeat and its flanking regions. About 500 nucleotides in a single strand of DNA of the 3.36 kb *Eco*RI fragment of the SCA1 gene shown in Figure 1 is represented. The locations of PCR primers are shown by solid lines with arrowheads.

Figure 4. Summary of SCA1 recombination events that led to the precise mapping of the SCA1 locus. Recombinant disease-carrying chromosomes are shown for the markers shown above. A schematic diagram of the relevant region of 6p22 (not drawn to scale) is shown at the top of the figure. Families are coded as follows: TX = Houston, MN = Minnesota, MI = Michigan, IT = Italy. Each recombination event is given a number following the family code.

Figure 5. Regional localization of 6p22-p23 STSs by PCR analysis of radiation reduced hybrids. Three panels (a-c) demonstrate the regional localization of D6S274, D6S288, and AM10GA. In each panel PCR amplification results are shown for genomic DNA, the I-7 cell line which retains 6p, the radiation reduced hybrids R17, R72, R86, and R54, and RJK88 hamster DNA. A blank control (c) is shown for every panel. R86 has been previously shown to retain D6S89; R17 and R72 are known to contain D6S88 and D6S108, two DNA markers which map centromeric to D6S89. An amplification product is seen in I-7, R17, R72, and R86 for D6S274 and D6S288, whereas the amplification product for AM10GA is only seen in I-7 and R86 confirming that D6S274 and D6S288 map centromeric to AM10GA and D6S89.

Figure 6. A schematic diagram of 6p22-p23 region showing the new markers and the YAC contig. At the bottom of the diagram, the radiation hybrid reduced panel used for regional mapping is shown. YAC clones are represented as dark lines, open segments indicate a noncontiguous region of DNA. The discontinuity shown in YAC clone 351B10 indicate that this YAC has an internal deletion. All of the ends of the YAC clones that were isolated are designated by an "L" for the left end or an "R" for the right end.

Figure 7. Genotypic data for 6p22-p23 dinucleotide repeat markers are shown for a reduced pedigree from the MN-SCA1 kindred. This figure summarizes a second recombination event that led to the precise mapping of the SCA1 locus.

Figure 8. Long-range restriction maps of YACs, 227B1, 60H7, 195B5, A250D5, and 379C2. YACs 351B10, 172B5, 172B5, and 168F1 were also used in the restriction analysis (data not shown). The restriction sites are marked as N, *Not*I; B, *Bss*HII; Nr, *Nru*I; M, *Mlu*I, S, *Sac*II, and Sa, *Sal*I. A summary map of the SCA1 gene region with the position of the DNA markers used as probes (boxes) is shown. The centromere-telomere orientation is indicated by cen/tel respectively.

Figure 9. Physical map of the SCA1 region. The positions of various genetic markers and sequence tagged sites (STSs) relative to the overlapping YAC clones are shown. AM10 and FLB1 are STSs developed using a radiation reduced hybrid retaining chromosome 6p22-p23, A205D5-L and 195B5-L are STSs from insert termini of YACs A250D5 and 195B5. D6S89, D6S109, D6S288 and D6S274, and AM10-GA are dinucleotide repeat markers used in the genetic analysis

of SCA1 families. The SCA1 candidate region is flanked by the D6S274 and D6S89 markers which identify the closest recombination events. The YAC clones shown here are indicated by the cross-hatched markings. YAC 172B5 has two non-contiguous segments of DNA as indicated by the open bar for the non-6p segment. The YACs are designated according to St. Louis and CEPH libraries. The position of the cosmid contig (C) which contains the overlapping cosmids which are $(CAG)_n$ positive is indicated by a solid black bar. The overlap between the YACs was determined by long-range restriction analysis. Orientation is indicated as centromeric (Cen) and telomeric (Tel).

Figure 10. Southern blot analysis of leukocyte DNA using the 3.36-kb *Eco*RI fragment which contains the repeat as a probe. **Figure 10a:** TaqI-digested DNA from a TX-SCA1 kindred. The unaffected spouse has a single fragment at 2830-bp. The affected individual with onset at 25 years of age has the 2830-bp fragment as well as a 2930-bp fragment. The affected child with onset at 4 years inherited the normal 2830-bp from her mother, and has a new fragment of 3000-bp not seen in either parent. **Figure 10b:** TaqI-digested DNA from individuals from a MN-SCA1 kindred. The unaffected spouse and the unaffected sibling have a 2830-bp fragment. The two affected brothers have the 2830-bp fragment as well as an expanded fragment of 2900-bp in the sib with onset at 25 years and 2970-bp in the sib with onset at 9 years. **Figure 10c:** BstNI-digested DNA from the TX-SCA1 kindred. Lanes 1-3 are from the same kindred depicted in (A). The normal fragment size is 530-bp, in individuals with onset at 25-30 years (lanes 1 and 4) the fragment expands to 610-bp. In the individual with onset at 15 years of age (lane 7) the fragment size is 640-bp, and in the individual with onset at 4 years (lane 3) the fragment size is 680-bp. The DNA in lane 5 is from a 14 year old child who is asymptomatic.

Figure 11. Analysis of the PCR-amplified products containing the trinucleotide repeat tract in normal and SCA1 individuals. The CAG-a/CAG-b primer pair was used in panel (a) whereas the Rep-1/Rep-2 primer pair was used in panel (b). The individuals in lanes 1, 2 and 3 in panel (a) are brothers. The range for the normal (NL) and expanded (EXP) $(CAG)_n$ repeat units is indicated.

Figure 12. A scatter plot for the age-at-onset in years versus the number of the $(CAG)_n$ repeat units is shown to demonstrate the correlation between the age-at-onset and the size of the expansion. A linear correlation coefficient of

-0.845 was obtained. In addition a curvilinear correlation coefficient was calculated given the non-linear pattern of the plot. The curvilinear correlation coefficient is -0.936.

Figure 13. Schematic representation of the SCA1 cDNA contig. A subset of overlapping phage cDNA clones (black bars) and 5′-RACE-PCR product (R1) spanning 10.66 kb of the SCA1 transcript is shown. cDNA clone 31-5 contains the entire coding region for the SCA1 gene product, ataxin-1. On top, a schematic shows the structure of the SCA1 transcript; the sizes of the coding region (rectangle) as well as the 5′UTR and the 3′UTR (thin lines) are indicated. The position of the CAG repeat within the coding region is also shown. An asterisk indicates the clones used as probes to screen the cDNA libraries. At the bottom the positions of *Bam*HI (B), *Hind*III (H), and *Taq*I (T) restriction sites are shown.

Figure 14. Northern blot analysis of the SCA1 gene using RNAs from multiple human tissues. The panel on the left is probed with a PCR product from a portion of the coding region (bp 2460 to bp 3432). The panel on the right is hybridized with the 3J cDNA clone from the 3′UTR. An ~11 kb transcript is detected in RNAs from all tissues using both probes as well as the cDNA clones 31-5 and 8-8, both of which contain the CAG repeat (Figure 13).

Figure 15. The sequence of the SCA1 transcript. The sequences of primers 9b, 5F and 5R (bp 129-147, bp 173-191 and bp 538-518 respectively in the 5′ to 3′ orientation) are underlined. The protein sequence encoded by the DNA is shown below the DNA sequence. The CAG repeat region is from about bp 1524 to about bp 1613.

Figure 16. ***a.*** The structure of the SCA1 transcript and the various splice variants. The schematic on top represents the nine exons (not drawn to scale) and their respective sizes. The stippled areas indicate the coding region. The structure of five cDNA clones representing different splice variants of the SCA1 transcript are also shown. Clones 8-8 and 8-9b are phage clones, RT-PCR1 and RT-PCR2 are two clones obtained by RT-PCR carried out on cerebellar poly-$(A)^+$ RNA using the primers 9b and 5R (Figure 15). Only 30 bp of exon 1 were present in clone 8-9b and RT-PCR products as indicated by the broken line in the rectangles. ***b.*** Detection of alternative splicing of the SCA1 transcript in cerebellar poly-$(A)^+$ RNA (CBL RNA). RT-PCR analysis was carried out using two sets of primers: 9b-5R and 5F-5R. PCR products of the expected size were detected in

CBL RNA in the presence of reverse transcriptase (+RT) with both pairs of primers. Using the 9b-5R pair at least two larger PCR products were also detected. Using the 5F-5R pair for RT-PCR at annealing T < 60°, some faint bands in the same size range as those seen using the 9b-5R primer pair were also seen. 8-8 and 8-9b are the phage clones used as positive controls. The sizes of the relevant bands of the molecular weight marker (FX174 cut with *Hae*III) are indicated on the left.

Figure 17. Intron-exon boundaries of the SCA1 gene. Splice acceptor and splice donor sites are indicated in bold letters. The numbers at the beginning and the end of each exon refer to the position in the composite sequence of SCA1 in Figure 15. Uppercase letters indicate exon sequences, lowercase letters indicate intron sequences. Y= pyrimidine; R= purine; N= undefined.

Figure 18. Genomic structure of the SCA1 gene. The nine exons of the SCA1 gene (solid rectangles not drawn to scale) were localized based on the restriction map of the SCA1 region by Southern analysis using rare cutter DNA digests from several YAC clones. A representative map using YAC clone 227B1, which encompasses the SCA1 gene, is shown. The restriction map of this YAC has been confirmed by analysis of four overlapping YAC clones in the region. The centromere-telomere orientation is indicated by CEN-TEL, respectively. L= left YAC end; R= right YAC end; B= *Bss*HII; C= *Csp*I; M= *Mlu*I; N= *Not*I; Nr= *Nru*I; S= *Sac*II.

Figure 19. Analysis of expression of the expanded SCA1 allele. RT-PCR was carried out on lymphoblast poly-$(A)^+$RNA from one unaffected individual (lane 1) and four SCA1 patients (lanes 2 through 5) using primers Rep1 and Rep2. This analysis shows that both the normal and the expanded SCA1 alleles are transcribed. The number of the repeat units for each allele is indicated below each lane; lane 6 is the RT minus control.

Figure 20. Distributions of CAG repeat lengths from unaffected control individuals and from SCA1 alleles. Normal alleles range in size from 19 to 36 repeat units while disease alleles contain from 42 to 81 repeats.

Detailed Description

Substantial efforts have been made to localize the SCA1 gene using genetic and physical mapping methods. Genetically, SCA1 is flanked on the centromeric side by D6S88 at a r*Eco*mbination fraction of approximately 0.08 (based on marker-marker distances using the Centre d'Etude du Polymorphisme Humain (CEPH) reference families) and on the telomeric side by F13A at a recombination fraction of 0.19. See, L.P.W. Ranum et al., Am. J. Hum. Genet., 49, 31-41 (1991). Both markers are quite distant and are not practical for use in efforts aimed at cloning the SCA1 gene. The D6S89 marker maps closer to the SCA1 gene.

To localize SCA1 more precisely, five dinucleotide polymorphisms near D6S89 have been identified. A new marker, AM10GA, demonstrates no recombination with SCA1. Linkage analysis and analysis of recombination events confirm that SCA1 maps centromeric to D6S89 with D6S109 as the other flanking marker at the centromeric end and establishes the following order: centromere-D6S109-AM10GA/SCA1-D6S89-LR40-D6S202-telomere. The genetic distance between the two flanking markers D6S109 and D6S89 is about 6.7 cM based on linkage analysis using 40 reference families from the Centre d'Etude du Polymorphisme Humain (CEPH).

A. SCA1 Gene and Method of Diagnosis

The size of the candidate region on the short arm of chromosome 6 containing the SCA1 locus is about 1.2 Mb, and is flanked by D6S274 to the centromeric side and D6S89 to the telomeric side. The SCA1 gene spans 450 kb of genomic DNA and is organized in nine exons (Figure 15 is representative of the SCA1 gene from a normal individual). The SCA1 transcript (i.e., mRNA or cDNA clone) is about 10.6-11 kb. The gene is transcribed in both normal and affected SCA1 alleles. The structure of the gene is unusual in that it contains seven exons in the 5'-untranslated region, two large exons (2080 bp and 7805 bp) which contain a 2448-bp coding region, and a 7277 bp 3'-untranslated region. The first four non-coding exons undergo extensive alternative splicing in several tissues.

The gene for SCA1 contains a highly polymorphic CAG repeat that is located within a 3.36-kb fragment produced by digestion of the candidate region with the restriction enzyme, *Eco*RI. The CAG repeat region preferably lies within

the coding region and codes for polyglutamine. This region of CAG repeating sequences is unstable and expanded in individuals with SCA1. Southern and PCR analyses of the $(CAG)_n$ repeat demonstrate a correlation between the size of the repeat expansion and the age-at-onset of SCA1 and severity of the disorder. That is, individuals with more repeat units (or longer repeat tracts) tend to have both an early age of onset and a more severe disease coarse. These results demonstrate that SCA1, like fragile X syndrome, myotonic dystrophy, X-linked spinobulbar muscular atrophy, and Huntington disease, displays a mutational mechanism involving expansion of an unstable trinucleotide repeat.

The identification of a trinucleotide repeat expansion associated with SCA1 allows for improved diagnosis of the disease. Thus, in addition to being directed to the gene for SCA1 and the protein encoded thereby, the present invention also relates to methods of diagnosing SCA1. These diagnostic methods can involve any known method for detecting a specific fragment of DNA. These methods can include direct detection of the DNA or indirect through detection of RNA or proteins, for example. For example, Southern or Northern blotting hybridization techniques using labeled probes can be used. Alternatively, PCR techniques can be used with novel primers that amplify the CAG repeating region of the *Eco*RI fragment. Nucleic acid sequencing can also be used as a direct method of determining the number of CAG repeats.

For example, DNA probes can be used for identifying DNA segments of the affected allele of the SCA1 gene. DNA probes are segments of labeled, single-stranded DNA which will hybridize, or noncovalently bind, with complementary single-stranded DNA derived from the gene sought to be identified. The probe can be labeled with any suitable label known to those skilled in the art, including radioactive and nonradioactive labels. Typical radioactive labels include ^{32}P, ^{125}I, ^{35}S, and the like. Nonradioactive labels include, for example, ligands such as biotin or digoxigenin as well as enzymes such as phosphatase or peroxidases, or the various chemiluminescers such as luciferin, or fluorescent compounds like fluorescein and its derivatives. The probe may also be labeled at both ends with different types of labels for ease of separation, as, for example, by using an isotopic label at one end and a biotin label at the other end.

Using DNA probe analysis, the target DNA can be derived by the enzymatic digestion, fractionation, and denaturation of genomic DNA to yield a

complex mixture incorporating the DNA from many different genes, including DNA from the short arm of chromosome 6, which includes the SCA1 locus. A specific DNA gene probe will hybridize only with DNA derived from its target gene or gene fragment, and the resultant complex can be isolated and identified by techniques known in the art.

In general, for detecting the presence of a DNA sequence located within the SCA1 gene, the genomic DNA is digested with a restriction endonuclease to obtain DNA fragments. The source of genomic DNA to be tested can be any biological specimen that contains DNA. Examples include specimen of blood, semen, vaginal swabs, tissue, hair, and body fluids. The restriction endonuclease can be any that will cut the genomic DNA into fragments of double-stranded DNA having a particular nucleotide sequence. The specificities of numerous endonucleases are well known and can be found in a variety of publications, e.g. Maniatis et al.; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory: New York (1982). That manual is incorporated herein by reference in its entirety. Preferred restriction endonuclease enzymes include *Eco*RI, *Taq*I, and *Bst*NI. *Eco*RI is particularly preferred.

Diagnosis of the disease can alternatively involve the use of the polymerase chain reaction sequence amplification method (PCR) using novel primers. U.S. Patent No. 4,683,195 (Mullis et al., issued July 28, 1987) describes a process for amplifying, detecting and/or cloning nucleic acid sequences. The method involves treating extracted DNA to form single-stranded complementary strands, treating the separate complementary strands of DNA with two oligonucleotide primers, extending the primers to form complementary extension products that act as templates for synthesizing the desired nucleic acid molecule; and detecting the amplified molecule. More specifically, the method steps of treating the DNA with primers and extending the primers include the steps of: adding a pair of oligonucleotide primers, wherein one primer of the pair is substantially complementary to part of the sequence in the sense strand and the other primer of each pair is substantially complementary to a different part of the same sequence in the complementary antisense strand; annealing the paired primers to the complementary molecule; simultaneously extending the annealed primers from a 3' terminus of each primer to synthesize an extension product complementary to the strands annealed to each primer wherein said extension products after separation

from the complement serve as templates for the synthesis of an extension product for the other primer of each pair; and separating said extension products from said templates to produce single-stranded molecules. Variations of the method are described in U.S. Patent No. 4,683,194 (Saiki et al., issued July 28, 1987). The polymerase chain reaction sequence amplification method is also described by Saiki et al., Science, 230, 1350-1354 (1985) and Scharf et al., Science, 324, 163-166 (1986). The discussion of the these techniques in each of these references is incorporated herein by reference.

The primers are oligonucleotides, either synthetic or naturally occurring, capable of acting as a point of initiating synthesis of a product complementary to the region of the DNA sequence containing the CAG repeating trinucleotides of the SCA1 locus of the short arm of chromosome 6. The primer includes a nucleotide sequence substantially complementary to a portion of a strand of an affected or a normal allele of a fragment (preferably a 3.36 kb *Eco*RI fragment) of an SCA1 gene having a $(CAG)_n$ region. The primer sequence has at least about 11 nucleotides, preferably at least about 16 nucleotides and no more than about 35 nucleotides. The primers are chosen such that they produce a primed product of about 70-350 base pairs, preferably about 100-300 base pairs. More preferably, the primers are chosen such that nucleotide sequence is substantially complementary to a portion of a strand of an affected or a normal allele within about 150 nucleotides on either side of the $(CAG)_n$ region, including directly adjacent to the $(CAG)_n$ region.

Examples of preferred primers are shown by solid lines with arrowheads in Figure 3. The primers are thus selected from the group consisting of CCGGAGCCCTGCTGAGGT (CAG-a), CCAGACGCCGGGACAC (CAG-b), AACTGGAAATGTGGACGTAC (Rep-1), CAACATGGGCAGTCTGAG (Rep-2), CCACCACTCCATCCCAGC (GCT-435), TGCTGGGCTGGTGGGGGG (GCT-214), CTCTCGGCTTTCTTGGTG (Pre-1), and GTACGTCCACATTTCCAGTT (Pre-2). These primers can be used in various combinations or with any other primer that can be designed to hybridize to a portion of DNA of a fragment (preferably a 3.36 kb *Eco*RI fragment) of an SCA1 gene having a CAG repeat region. For example, the primer labeled Rep-2 can be combined with the primer labeled CAG-a, and the primer labeled CAG-b can be combined with the primer labeled Rep-1. More preferably the primers are the sets of primer pairs designed as

CAG-a/CAG-b, Rep-1/Rep-2, Rep-1/GCT-435, for example. These primer sets successfully amplify the CAG repeat units of interest using PCR technology. Alternatively, they can be used in various known techniques to sequence the SCA1 gene.

As stated previously, other methods of diagnosis can be used as well. They can be based on the isolation and identification of the repeat region of genomic DNA (CAG repeat region), cDNA (CAG repeat region), mRNA (GUC repeat region), and protein products (glutamine repeat region). These include, for example, using a variety of electrophoresis techniques to detect slight changes in the nucleotide sequence of the SCA1 gene. Further nonlimiting examples include denaturing gradient electrophoresis, single strand conformational polymorphism gels, and nondenaturing gel electrophoresis techniques.

The mapping and cloning of the SCA1 gene allows the definitive diagnosis of one type of the dominantly inherited ataxias using a simple blood test. This represents the first step towards an unequivocal molecular classification of the dominant ataxias. A simple and reliable classification system for the ataxias is important because the clinical symptoms overlap extensively between the SCA1 and the non-SCA1 forms of the disease. Furthermore, a molecular test for the only known SCA1 mutation permits presymptomatic diagnosis of disease in known SCA1 families and allows for the identification of sporadic or isolated CAG repeat expansions where there is no family history of the disease. Thus, the present invention can be used in family counseling, planning medical treatment, and in standard work-ups of patients with ataxia of unknown etiology.

B. Cloning

Cloning of SCA1 DNA into the appropriate replicable vectors allows expression of the gene product, ataxin-1, and makes the SCA1 gene available for further genetic engineering. Expression of ataxin-1 or portions thereof, is useful because these gene products can be used as antigens to produce antibodies, as described in more detail below.

1. Isolation of DNA

DNA containing the SCA1 gene may be obtained from any cDNA library prepared from tissue believed to possess the SCA1 mRNA and to express it

at a detectable level. Preferably, the cDNA library is from human fetal brain or adult cerebellum. Optionally, the SCA1 gene may be obtained from a genomic DNA library or by *in vitro* oligonucleotide synthesis from the complete nucleotide or amino acid sequence.

Libraries are screened with appropriate probes designed to identify the gene of interest or the protein encoded by it. Preferably, for cDNA libraries, suitable probes include oligonucleotides that consist of known or suspected portions of the SCA1 cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments thereof that consist of the same or a similar gene. Optionally, for cDNA *expression* libraries (which express the protein), suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to the SCA1 gene product, ataxin-1. Appropriate probes for screening *genomic* DNA libraries include, but are not limited to, oligonucleotides, cDNAs, or fragments thereof that consist of the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected probe may be accomplished using standard procedures.

Screening cDNA libraries using synthetic oligonucleotides as probes is a preferred method of practicing this invention. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous to minimize false positives. The actual nucleotide sequence(s) of the probe(s) is usually designed based on regions of the SCA1 gene that have the least codon redundancy. The oligonucleotides may be degenerate at one or more positions, i.e., two or more different nucleotides may be incorporated into an oligonucleotide at a given position, resulting in multiple synthetic oligonucleotides. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide can be labeled such that it can be detected upon hybridization to DNA in the library being screened. A preferred method of labeling is to use ATP and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Of particular interest is the SCA1 nucleic acid that encodes a full-length mRNA transcript, including the complete coding region for the gene product,

ataxin-1. Nucleic acid containing the complete coding region can be obtained by screening selected cDNA libraries using the deduced amino acid sequence.

An alternative means to isolate the SCA1 gene is to use PCR methodology. This method requires the use of oligonucleotide primer probes that will hybridize to the SCA1 gene. Strategies for selection of PCR primer oligonucleotides are described below.

2. Insertion of DNA into Vector

The nucleic acid (e.g., cDNA or genomic DNA) containing the SCA1 gene is preferably inserted into a replicable vector for further cloning (amplification of the DNA) or for expression of the gene product, ataxin-1. Many vectors are available, and selection of the appropriate vector will depend on: 1) whether it is to be used for DNA amplification or for DNA expression; 2) the size of the nucleic acid to be inserted into the vector; and 3) the host cell to be transformed with the vector. Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organism but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome. Each replicable vector contains various structural components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. These components are described in detail below.

Construction of suitable vectors employs standard ligation techniques known in the art. Isolated plasmids or DNA fragments are cleaved, tailored, and relegated in the form desired to generate the plasmids required. Typically, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants are selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by methods known in the art. See, e.g., Messing et al., Nucl. Acids Res., 9, 309 (1981) and Maxam et al., Methods in Enzymology, 65, 499 (1980).

Optionally, DNA may also be amplified by direct insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a

sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of SCA1 DNA. However, the recovery of genomic DNA containing the SCA1 gene is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the SCA1 DNA.

Replicable cloning and expression vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter and a transcription termination sequence.

Vector component: signal sequence. A signal sequence may be used to facilitate extracellular transport of a cloned protein. To this end, the SCA1 gene product, ataxin-1, may be expressed not only directly, but also as a fusion product with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the cloned protein or polypeptide. The signal sequence may be a component of the vector, or it may be a part of the SCA1 DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells, a prokaryotic signal sequence may be selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp or heat-stable intertoxin II leaders. For yeast secretion the signal sequence used may be, for example, the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression, a native signal sequence may be satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

Vector component: origin of replication. Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2m plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian

cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Vector component: marker gene. Expression and cloning vectors may contain a marker gene, also termed a selection gene or selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that: (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, streptomycin or tetracycline; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the SCA1 nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only transformants are uniquely adapted to survive by virtue of having taken up the marker. For example, cells transformed with the DHFR selection gene are first identified by culturing all the transformants in a culture medium that contains methotrexate, a competitive antagonist for DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77, 4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of the other DNA comprising the expression vectors, such as the SCA1 gene. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to methotrexate is employed. Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with SCA1 DNA, wild-type DHFR protein, and another selectable marker such as

aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in a medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin or neomycin. A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282, 39 (1979); Kingsman et al., Gene, 7, 141 (1979); or Tschemper et al., Gene, 10, 157 (1980)). The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC NO. 44076 or PEP4-1 (Jones, Genetics, 85, 12 (1977)). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu*2 deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu*2 gene.

Vector component: promoter. Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the SCA1 nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as the ataxin-1 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. In contrast, constitutive promoters produce a constant level of transcription of the cloned DNA segment.

At this time a large number of promoters recognized by a variety of potential host cells are well known in the art. Promoters are removed from their source DNA using a restriction enzyme digestion and inserted into the cloning vector using standard molecular biology techniques. Both the native SCA1 promoter sequence and many heterologous promoters can be used to direct amplification and/or expression of the SCA1 DNA. Heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed protein as compared to the native promoter. Well-known promoters suitable for use with prokaryotic hosts include the beta-lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (trp) promoter system, and

hybrid promoters such as the tac promoter. Such promoters can be ligated to SCA1 DNA using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems may contain a Shine-Dalgarno sequence for RNA polymerase binding.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bp upstream from the site where transcription is initiated Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is the CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be a signal for addition of the poly A tail to the 3' end of the coding sequence. All these sequences are suitably inserted into eukaryotic expression vectors. Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase and glucokinase. Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization.

SCA1 transcription from vectors in mammalian host cells can be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, Hepatitis-B virus and most preferably Simian Virus 40 (SV40) (Fiers et al., Nature, 273, 113 (1978); Mulligan et al., Science, 209, 1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78, 7398-7402 (1981)). Heterologous mammalian promoters (e.g., the actin promoter or an immunoglobulin promoter) and heat-shock promoters can also be used, as can the promoter normally associated with the SCA1 sequence itself, provided such promoters are compatible with the host cell systems.

Vector component: enhancer element. Transcription of SCA1 DNA by higher eukaryotes can be increased by inserting an enhancer sequence into the

vector. Enhancers are *cis*-acting elements of DNA, usually having about 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation- and position-independent, having been found 5' and 3' to the transcription unit, within an intron as well as within the coding sequence itself. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-fetoprotein, and insulin). Typically, however, an enhancer from a eukaryotic cell virus will be used. Examples include the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the SCA1 gene, but is preferably located at a site 5' of the promoter.

Vector component: transcription termination. Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) can also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally, 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions can contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of mRNA encoding ataxin-1.

Preferably, the pMAL™-2 vectors (New England Biolabs, Beverly, MA) are used to create the expression vector. These vectors provide a convenient method for expressing and purifying ataxin-1 produced from the cloned SCA1 gene. The SCA1 gene is inserted downstream from the *malE* gene of *E. coli*, which encodes maltose-binding protein (MBP) resulting in the expression of an MBP fusion protein. The method uses the strong "tac" promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences, and a one-step purification of the fusion protein using MBP's affinity for maltose. The vectors express the *malE* gene (with or without its signal sequence) fused to the *lacZα* gene. Restriction sites between *malE* and *lacZα* are available for inserting the coding sequence of interest. Insertion inactivates the β-galactosidase α-fragment activity of the *malE-lacZα* fusion, which results in a blue to white color change on Xgal plates when the construction is transformed into an α-complementing host such as TB1 (T.C. Johnston et al., J. Biol. Chem., 261, 4805-4811 (1986)) or JM107 (C. Yanisch-Perron et al., Gene, 33, 103-119 (1985)). When present, the signal peptide on pre-

MBP directs fusion proteins to the periplasm. For fusion proteins that can be successfully exported, this allows folding and disulfide bond formation to take place in the periplasm of *E. coli*, as well as allowing purification of the protein from the periplasm. The vectors carry the lac^q gene, which codes for the Lac repressor protein. This keeps expression from P_{lac} low in the absence of isopropyl β-D-thiogalactopyranoside (IPTG) induction. The pMAL™-2 vectors also contain the sequence coding for the recognition site of the specific protease factor Xa, located just 5' to the polylinker insertion sites. This allows MBP to be cleaved from ataxin-1 after purification. Factor Xa cleaves after its four amino acid recognition sequence, so that few or no vector derived residues are attached to the protein of interest, depending on the site used for cloning.

Also useful are expression vectors that provide for transient expression in mammalian cells of SCA1 DNA. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of ataxin-1 that have wild-type or variant biological activity.

3. Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryotic cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcsecans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, *in vitro* methods of cloning, *e.g.*, PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for SCA1-encoding vectors. *Saccaromyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccaromyces pombe, Kluyveromyces* hosts such as, *e.g., K. lactis, K. fragilis, K. bulgaricus, K. thermotolerans,* and *K. marxianus, yarrowia, Pichia pastoris, Candida, Trichoderma reesia, Neurospora crassa*, and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans*.

Suitable host cells for the expression of glycosylated ataxin-1 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, *e.g.,* Luckow et al., Bio/Technology, 6, 47-55 (1988); Miller et al., Genetic Engineering, 8, 277-279 (1986); and Maeda et al., Nature, 315, 592-594 (1985). A variety of viral strains for transfection are publicly available, *e.g.,* the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the SCA1 DNA. During incubation of the plant cell culture with *A. tumefaciens*, the SCA1 DNA is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the SCA1 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., J. Mol. Appl. Gen., 1, 561 (1982).

Vertebrate cells can also be used as hosts. Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years.

Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (CAS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol., 36, 59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77, 4216 (1980)); mouse sertoli cells (TM4, Mather, Biol Reprod., 23, 243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL 51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383, 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

4. Transfection and transformation

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequence are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, the calcium phosphate precipitation method and electroporation are commonly used. Successful transfection is generally recognized when any indication of the operation of the vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. Calcium chloride is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* can be used for transformation of certain plant cells. For mammalian cells without cell walls, the calcium phosphate precipitation method of Graham et al., Virology, 52, 456-457 (1978) is preferred. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J.

Bact., 130, 946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 78 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

5. Cell Culture

Prokaryotic cells used to produce the SCA1 gene product, ataxin-1, are cultured in suitable media, as described generally in Sambrook et al. The mammalian host cells used to produce the SCA1 gene product may be cultured in a variety of media. Commercially available media such as Hams F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing the host cells. These media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The host cells referred to in this disclosure encompass in *in vitro* culture as well as cells that are within a host animal.

C. Protein

The SCA1 gene encodes a novel protein, ataxin-1, a representative example of which is shown in Figure 15 with an estimated molecular weight of about 87 kD. It is to be understood that ataxin-1 represents a set of proteins produced from the SCA1 gene with its unstable CAG region. Ataxin-1 can be produced from cell cultures. With the aid of recombinant DNA techniques, synthetic DNA and cDNA coding for ataxin-1 can be introduced into microorganisms which can then be made to produce the peptide. It is also possible to manufacture ataxin-1 synthetically, in a manner such as is known for peptide syntheses.

Ataxin-1 is preferably recovered from the culture medium as a cytosolic polypeptide, although it can also be recovered as a secreted polypeptide when expressed with a secretory signal.

Ataxin-1 can be purified from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogenous as ataxin-1. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The ataxin-1 may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the ataxin-1 is membrane bound. If necessary, ataxin-1 is further purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; ligand affinity chromatography, using, e.g., protein A Sepharose columns to remove contaminants such as IgG.

Ataxin-1 variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native ataxin-1, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a ataxin-1 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal ataxin-1 column can be employed to absorb the ataxin-1 variant by binding it to at least one remaining immune epitope. Alternatively, the ataxin-1 may be purified by affinity chromatography using a purified ataxin-1-IgG coupled to a (preferably) immobilized resin such as Affi-Gel 10 (Bio-Rad, Richmond, CA) or the like, by means well-known in the art. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants.

Covalent modifications of ataxin-1 are included within the scope of this invention. Both native ataxin-1 and amino acid sequence variants of the ataxin-1 may be covalently modified. Covalent modifications included within the scope of

this invention are those producing one or more ataxin-1 fragments. Ataxin-1 fragments having any number of amino acid residues may be conveniently prepared by chemical synthesis, by enzymatic or chemical cleavage of the full-length or variant ataxin-1 polypeptide, or by cloning and expressing only portions of the SCA1 gene. Other types of covalent modifications of ataxin-1 or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of the ataxin-1 or fragments thereof with a derivatizing agent capable of reacting with selected side chains or the N- or C-terminal residues.

For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as iodoacetic acid or iodoacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, iodoacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, *p*-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate *p*-bromophenacyl. Lysinyl and amino terminal residues are derivatized with succinic or other carboxylic acid anhydrides and imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin, among others.

Specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepared labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl)carbodiimide. Furthermore, aspartyl and glutamyl

residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking ataxin-1 to a water-insoluble support matrix or surface for use in the method for purifying anti-ataxin-1 antibodies, and vice versa. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, and N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(*p*-azidophenyl)dithio]propiomidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, amidation of any C-terminal carboxyl group, and glycosylation of any suitable residue.

D. Antibodies

The present invention also relates to polyclonal or monoclonal antibodies raised against ataxin-1 or ataxin-1 fragments (preferably fragments having 8-40 amino acids, more preferably 10-20 amino acids, that form the surface of the folded protein), or variants thereof, and to diagnostic methods based on the use of such antibodies, including but not limited to Western blotting and ELISA (enzyme-linked immunosorbant assay).

Polyclonal antibodies to the SCA1 polypeptide generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of ataxin-1, ataxin-1 fragments, or variants thereof, and an adjuvant. The polypeptide can be a

cloned gene product or a synthetic molecule. Preferably, it corresponds to a position in the protein sequence that is on the surface of the folded protein and is thus likely to be antigenic. It may be useful to conjugate the SCA1 polypeptide (including fragments containing a specific amino acid sequence) to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehye, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

The route and schedule of immunizing a host animal or removing and culturing antibody-producing cells are variable and are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the host animal, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines. Preferably, rabbits are used to raise antibodies against ataxin-1.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining about 10 μg to about 1 mg of ataxin-1 with about 2-3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. About one month later the animals are boosted with about 1/5 to about 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. About 7 to 14 days later animals are bled and the serum is assayed for anti-ataxin-1 polypeptide titer.

Serum antibodies (IgG) are purified via protein purification protocols that are well known in the art. Antibody/antigen reactivity is analyzed using Western blotting, wherein suspected antigens are blotted to a nitrocellulose filter, exposed to potential antibodies and allowed to hybridize under defined conditions. See Gershoni et al., Anal. Biochem., 131, 1-15 (1983). The protein antigens can

then be sequenced using standard sequencing methods directly from the antibody/antigen complexes on the nitrocellulose support.

Monoclonal antibodies are prepared by recovering immune cells - typically spleen cells or lymphocytes from lymph node tissue - from immunized animals (usually mice) and immortalizing the cells in conventional fashion, *e.g.*, by fusion with myeloma cells. The hybridoma technique described originally by Kohler et al., Eur. J. Immunol., 6, 511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens. It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody-producing cells and the myeloma be from the same species. While mouse monoclonal antibodies are routinely used, the present invention is not so limited. In fact, although mouse monoclonal antibodies are typically used, human antibodies may be used and may prove to be preferable. Such antibodies can be obtained by using human hybridomas. Cote et al.; Monoclonal Antibodies and Cancer Therapy; A.R. Liss, Ed.; p. 77 (1985).

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, *e.g.*, ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the ataxin-1 in test samples.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments), which bypass the generation of monoclonal antibodies, are encompassed within the practice of this invention. Antibody-specific messenger RNA molecules are extracted from immune system cells taken from an immunized animal, transcribed into complementary DNA (cDNA), and the cDNA is cloned into a bacterial expression system.

The anti-ataxin-1 antibody preparations of the present invention are specific to ataxin-1 and do not react immunochemically with other substances in a

manner that would interfere with a given use. For example, they can be used to screen for the presence of ataxin-1 in tissue extracts to determine tissue-specific expression levels of ataxin-1.

The present invention also encompasses an immunochemical assay that involves subjecting antibodies directed against ataxin-1 to reaction with the ataxin-1 present in a sample to thus form an (ataxin-1/anti-ataxin-1) immune complex, the formation and amount of which are measures - qualitative and quantitative, respectively - of the ataxin-1 presence in the sample. The addition of other reagents capable of biospecifically reacting with constituents of the protein/antibody complex, such as anti-antibodies provided with analytically detectable groups, facilitates detection and quantification of ataxin-1 in biological samples, and is especially useful for quantitating the level of ataxin-1 in biological samples. Ataxin-1/anti-ataxin-1 complexes can also be subjected to amino acid sequencing using methods well known in the art to determine the length of a polyglutamine region and thereby provide information about likelihood of affliction with spinocerebellar ataxia and likely age of onset. Competitive inhibition and non-competitive methods, precipitation methods, heterogeneous and homogeneous methods, various methods named according to the analytically detectable group employed, immunoelectrophoresis, particle agglutination, immunodiffusion and immunohistochemical methods employing labeled antibodies may all be used in connection with the immune assay described above.

The invention has been described with reference to various specific and preferred embodiments and will be further described by reference to the following detailed examples. It is understood, however, that there are many extensions, variations, and modifications on the basic theme of the present invention beyond that shown in the examples and detailed description, which are within the spirit and scope of the present invention.

Experimental Section

I. The Gene for SCA1 Maps Centromeric to D6S89

To confirm the position of SCA1 with respect to D6S89 and to identify closer flanking markers, two dinucleotide repeat polymorphisms D6S109 and D6S202 were used. Using YAC clones isolated in the D6S89 region, three additional dinucleotide repeat polymorphisms were identified, one of which (AM10GA) showed no recombination with SCA1 and confirmed that D6S89 is telomeric to SCA1. The dinucleotide repeat at D6S109 revealed six recombination events with SCA1 and determined D6S109 to be the other flanking marker at the centromeric end. Linkage analysis, physical mapping data as discussed below, and analysis of recombination events demonstrated that the order of markers is as follows: Centomere - D6S109 - AM10GA/SCA1 - D6S89 - SB1 - LR40 - D6S202 - Telomere.

A. Materials and Methods

1. SCA1 Kindreds

Nine large SCA1 families were used in the present study. Clinical findings and linkage data demonstrating that these families segregated SCA1 have been previously reported. See, J.F. Jackson et al., N. Engl. J. Med., 296, 1138-1141 (1977); B.J.B. Keats et al., Am. J. Hum. Genet., 49, 972-977 (1991); L.P.W. Ranum et al., Am. J. Hum. Genet., 49, 31-41 (1991); and H.Y. Zoghbi et al., Am. J. Hum. Genet., 49, 23-30 (1991). Analysis of polymorphisms at the loci D6S109, AM10GA, SB1, LR40, and D6S202 was performed on individuals from these kindreds.

The Houston (TX-SCA1) kindred included 106 individuals, of whom 57 (25 affected) were genotyped. See, H.Y. Zoghbi et al., Ann. Neurol., 23, 580-584 (1988). Patients symptomatic at the time of exam, as well as asymptomatic individuals who had both a symptomatic child and a symptomatic parent, were classified as "affected." In this kindred, a deceased individual previously assigned as affected (from family history data) was reassigned an unknown status after review of medical records. This reassignment eliminated what was previously thought to be a recombination event between SCA1 and D6S89 in the TX-SCA1 kindred. To maximize the amount of information available for linkage analysis, the two chromosomes 6 in somatic cell hybrids for 15 affected individuals and one

unaffected individual from the TX-SCA1 kindred were separated. See, H.Y. Zoghbi et al., Am. J. Hum. Genet., 44, 255-263 (1989). The Louisiana (LA-SCA1) kindred included 50 individuals of whom 26 (8 affected) were genotyped. See, B.J.B. Keats et al., Am. J. Hum. Genet., 49, 972-977 (1991). The Minnesota (MN-SCA1) kindred included 175 individuals, of whom 106 (17 affected) were genotyped. See, J.L. Haines et al., Neurology, 34, 1542-1548 (1984); and L.P.W. Ranum et al., Am. J. Hum. Genet., 49, 31-41 (1991). The Michigan (MI-SCA1) kindred included 201 individuals, of whom 127 (25 affected) were genotyped. See, H.E. Nino et al., Neurology, 30, 12-20 (1980). The Mississippi (MS-SCA1) kindred included 84 individuals, of whom 37 (17 affected) were genotyped. See, J.F. Jackson et al., N. Engl. J. Med., 296, 1138-1141 (1977).

Four Italian families segregating SCA1 were analyzed; their clinical phenotype and HLA linkage data were reported previously. See, M. Spadaro et al., Acta Neurol. Scand., 85, 257-265 (1992). Three families originated in the Calabria Region (Southern Italy): family IT-P with 135 members of whom 80 (21 affected) were genotyped; for computational reasons, the family was subdivided into 3 different pedigrees (RM, VI, and FB) and only one of the 3 consanguinity loops was considered; family IT-NS, with 43 members of whom 27 (7 affected) were typed; family IT-NS with 51 members of whom 16 (3 affected) were typed. The fourth family, IT-MR, originated from Latium and consisted of 17 individuals of whom 10 (4 affected) were genotyped.

2. CEPH Families

The 40 CEPH reference families were genotyped at the D9S109, LR40 and D6S202 loci in order to provide a large number of informative meioses for marker-marker linkage analyses. Markers AM10GA and SB1 flank D6S89, having been isolated from a yeast artificial chromosome (YAC) contig built bidirectionally from D6S89 (see below). A subset of 18 CEPH families which defined 26 recombinants between D6S109 and D6S89 was genotyped at AM10GA and SB1 in order to determine the order of AM10GA, D6S89 and SB1 with respect to D6S109.

3. Cloning of Sequences Containing Dinucleotide Repeats

The identification and description of polymorphic dinucleotide repeats at the D6S109 and D6S202 loci have been previously reported. See, L.P.W. Ranum

et al., Nucleic Acids Res., 19, 1171 (1991); and F. LeBorgne-Demarquoy et al., Nucleic Acids Res., 19, 6060 (1991).

DNA fragments containing dinucleotide repeats were cloned at LR40 and SB1 from yeast artificial chromosome (YAC) clones at the LR40 and FLB1 loci, respectively (see below). DNA from each YAC clone was amplified in a 50 µl reaction containing 20 ng DNA, a single Alu primer (see below), 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.25 mM $MgCl_2$, 200 or 250 µM dNTPs, 0.01% (w/v) gelatin, and 1.25 units Thermus aquaticus DNA polymerase (Taq polymerase--Perkin Elmer, Norwalk, CT). For amplification of FLB1 YAC DNA, a primer complementary to the 5' end of the Alu consensus sequence (Oncor Laboratories, Gaithersberg, MD), designated SAL1, was used = 5'-AGGAGTGAGCCACCGCACCCAGCC-3' at a final concentration of 0.6 µM. For amplification of LR40 YAC DNA, 0.2 µM primer PDJ34 was used. See, C. Breukel et al., Nucleic Acids Res., 18, 3097 (1990). Samples were overlaid with mineral oil, denatured at 94°C for 5 minutes, then subjected to 30 cycles of 1 minute 94°C denaturation, 1 minute 55°C annealing, and 5 minutes 72°C extension. The last extension step was lengthened to 10 minutes. Electrophoresis of 15 µl of PCR products was performed on a 1.5% agarose gel, which was Southern blotted and hybridized with a probe prepared by random-hexamer-primed labelling of synthetic poly(dG-dT)-poly(dA-dC) (Pharmacia, Piscataway, NJ) using [α-^{32}P]dCTP, as described by A.P. Feinberg et al., Anal. Biochem., 137. 266-267 (1984). Fragments hybridizing with the dinucleotide repeat probe were identified and were subsequently purified by electrophoresis on a low-melt agarose gel. Fragments were excised and reamplified by PCR as above.

For LR40, reamplified DNA was repurified by low-melt gel electrophoresis, and DNA extracted from excised bands by passage through a glasswool spin column as described by D.M. Heery et al., Trends Genet., 6, 173 (1990). A purified 1.2-kb fragment was cloned into pBluescript plasmid modified as a "T-vector" as described by D. Marchuck et al., Nucleic Acids Res., 19, 1154 (1990). From this clone, a 0.6-kb *Hinc*II restriction fragment containing a GT repeat was subcloned into pBluescript plasmid, and sequenced on an Applied Biosystems, Inc. (Foster City, CA) automated sequencer.

For SB1, a reamplified 1-kb fragment was ethanol precipitated and blunt-end cloned into pBluescript plasmid. Plasmid DNA was isolated and PCR amplified in one reaction with M13 Reverse primer plus BamGT primer (5'-CCCGGATCCTGTGTGTGTGTGTGTG-3') and in a second reaction M13 Universal primer and BamCA primer (5'-CCCGGATCCACACACACACACACAC-3'). See, C.A. Feener et al., Am. J. Hum. Genet., 48, 621-627 (1991). PCR conditions were as above except primers were used at 1 μM concentration; 2.5 units Taq polymerase and approximately 30 ng DNA were used per reaction, with final reaction volumes of 100 μl, and an annealing temperature of 50°C. Products were precipitated, resuspended, and digested with *Bam*H1 (product of Universal primer reaction) or *Bam*H1 and *Hin*cII (product of Reverse primer reaction). These two fragments were cloned into pBluescript plasmid and sequenced as above.

Dinucleotide repeats were cloned at AM10 from a YAC containing this locus. A λFixII library was constructed using DNA from this yeast clone, and human clones were identified by filter hybridization using human placental DNA as a probe. A gridded array of these human clones was grown, and filters containing DNA from these clones were hybridized with a ^{32}P-labelled poly(dG-dT)-poly(dA-dC3) probe as described above. DNA was prepared from positive clones, digested with various restriction enzymes, and analyzed by agarose gel electrophoresis. Southern blotting and hybridization were carried out with the poly(dG-dT)-poly(dA-dC) probe. A 1-kb fragment hybridizing with the dinucleotide repeat probe was identified, clones into M13, and sequenced.

4. PCR Analysis

Primer sequences and concentrations, and PCR cycle times used for amplification of dinucleotide repeat sequences from human genomic DNA are presented in Table 1. For the LR40 polymorphism, primer set "A" was used for analysis of the TX-SCA1, LA-SCA1, and MS-SCA1 kindreds, while primer set "B" was used for all other kindreds. Buffer compositions were as follows: 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1.25 mM $MgCl_2$ (1.5 mM $MgCl_2$ for AM10GA), 250 μM dNTPs (200 μM dNTPs for AM10GA), 0.01% (w/v) gelatin, and 0.5 - 0.625 unit Taq polymerase. For the LR40 analysis, 2% formamide was included in the PCR buffer. When primer set B was used for LR40 analysis, 125 μM dNTPs, 1.5 mM

$MgCl_2$, and 1 unit Taq polymerase were used. All reaction volumes were 25 µl and contained 40 ng genomic DNA. Four microliters of each reaction was mixed with 2 µl formamide loading buffer, denatured at 90-100°C for 3 minutes, cooled on ice, and 2-4 µl was used for electrophoresis on a 4% or 6% polyacrylamide/7.65 M urea sequencing gel for 2-3 hours at 1100 V. PCR assay conditions have been reported previously for D6S202 and D6S109. See, L.P.W. Ranum et al., Nucleic Acids Res., 19, 1171 (1991); and F. LeBorgne-Demarquoy et al., Nucleic Acids Res., 19, 6060 (1991).

Table 1.
Primers and PCR conditions for amplification of dinucleotide repeat sequences

Marker/Type	Primers[a]	PCR Steps	PCR Cycles
AM10GA/$(GA)_n$	AAGTCAGCCTCTACTCTTTGT TGA	94°C for 30 sec.	
	CTTGGAGCAGTCTGTAGGGAG	55°C for 30 sec.	30
		72°C for 30 sec.	
SB1/$(GT)_n$	TGAAGTGATGTGCTCTGTTC	94°C for 60 sec.	
	AAAGGGGTAGAGGAAATGAG	60°C for 60 sec.	30
		72°C for 60 sec.	
LR40/$(GT)_n$	AGGAGAGGGGTCATGAGTTG	94°C for 60 sec.	
set A	GGCTCATGAATACATTACATG AAG	58°C for 60 sec.	25
		72°C for 60 sec.	
LR40/$(GT)_n$	CTCATTCACCTTAGAGACAAA TGGATAG	94°C for 60 sec.	
set B	ATGGTATAGGGATTTTNCCAA ACCTG	60°C for 60 sec.	27
		72°C for 45 sec.	

[a]Primers are shown as 5' to 3' sequence. The first primer of each pair was end-labelled with γ-^{32}P ATP and polynucleotide kinase. Primer concentrations were 1 mM.

5. SCA1 Linkage Analysis

The D6S109, AM10GA, D6S89, SB1, LR40 and D6S202 markers were analyzed for linkage to SCA1 using the computer program LINKAGE version 5.1 which includes the MLINK, ILINK, LINKMAP, CLODSCORE and CMAP programs. See, G.M. Lathrop et al., Proc. Natl. Acad. Sci. USA, 81, 3443-3446 (1984). Age dependent penetrance classes were assigned independently for each of the families included in the analysis. Marker alleles were recoded to reduce the number of alleles segregating in a family to four, five or six alleles to simplify the analysis. The allele frequencies for the various markers were based on the frequencies of the alleles among the spouses in each family and were determined separately for the two American black kindreds, for the Italian kindreds, and for the Caucasian kindreds from Minnesota, Michigan, and Mississippi, with the following exception - the allele frequencies for D6S109 in the MI and MN kindreds were based on the frequencies of the alleles in the CEPH families.

Maximum LOD scores for the various markers were calculated with the MLINK program by running each of the analyses separately for the various families, at theta values with increments of 0.0005 to 0.001, and then adding the values of each of the kindreds. The analyses were done separately to ensure that the allele frequencies for the various markers were representative for each of the ethnically diverse families. As a control, the recombination fractions at the maximum lod scores (Z_{max}) between each marker and SCA1 were calculated using the ILINK program after the allele frequencies for each marker were set equal to one another. In all cases the recombination frequencies were the same and Z_{max} values were very similar to those reported in Table 5 below.

6. CEPH Linkage Analysis

Forty CEPH families were typed for the GT repeat markers D6S109, D6S202 and LR40. The original alleles were recoded to five alleles. The SB1 and AM10 markers were typed in a subset of the CEPH panel which defined 26 recombinants from 18 different families between D6S109 and D6S89. The CLODSCORE program was used for the two-point analyses and CMAP was used for the three-and four-point analyses. For the three-point and four-point analyses, the interval between the mapped markers was fixed based on the two point $\theta_m = \theta_f$ results. The likelihood of the location of the test locus (SCA1) was calculated at 10

different positions within each interval. The test for sex difference in the Θ values was performed using a χ^2 statistic, with $\chi^2 = 2(\ln 10)[Z(\theta_m,\theta_f) - Z(\theta = \theta_m = \theta_f)]$, where $Z(\theta_m, \theta_f)$ is the overall Z_{max} for arbitrary θ_m and θ_f, while $Z(\theta = \theta_m = \theta_f)$ is the Z_{max} constrained to $\theta_m = \theta_f$. Under homogeneity (H1), χ^2 approximates a χ^2 with 1 d.f. Rejection of homogeneity occurs when $\chi^2 > 3.84$.

B. Results

1. Dinucleotide Repeat Cloning and Sequencing and Analysis

Dinucleotide repeats SB1 and LR40 were amplified directly from YAC clones by *Alu*-primed PCR and the dinucleotide repeat containing fragments were identified by hybridization. The PCR products were cloned either directly or by further amplification using tailed poly(GT) or poly(CA) primers paired with an Alu primer. In addition, two dinucleotide repeats were subcloned from a lambda phage clone from a library constructed from a YAC at the AM10 locus.

Dinucleotide repeats from the SB1, LR40, and AM10 loci were sequenced. At LR40, the cloned repeat sequence was $(CA)_{16}TA(CA)_{10}$. The AM10 fragment contained two repeat sequences separated by 45 bp of nonrepeat sequence. The first repeat, designated AM10GA, was $(GA)_2ATGACA(GA)_{11}$. The second repeat, designated AM10GT, was not used in this study because upon analysis of the TX-SCA1 kindred it yielded the same information as the AM10GA repeat. The AM10GT repeat consists of $(GA)_2AA(GA)_6GTGA(GT)_{16}AT(GT)_5$. Primer information for AM10GT is available through the Genome Data Base. At SB1, the repeat tract was not sequenced; only flanking sequence was determined.

As there are differences in allele distributions of markers among the different races, allele frequencies are reported here separately for the CEPH kindreds (Caucasian) and the TX-SCA1 kindred (American black) (Table 2). CEPH allele frequencies were based on 72 independent chromosomes for SB1, 82 independent chromosomes for AM10, and on the full set of 40 families for D6S109 and LR40. TX-SCA1 allele frequencies were based on 45 independent chromosomes for LR40, 43 independent chromosomes for SB1, 45 independent chromosomes for AM10, and 42 independent chromosomes for D6S109.

Table 2.

Allele frequencies of new markers

	D6S109[b]	AM10GA		SB1		LR40		D6S202[b]
Allele[a]	TXSCA1	TXSCA1	CEPH	TXSCA1	CEPH	TXSCA1	CEPH	TXSCA1
A_0	-	-	0.012	0.070	-	-	-	-
A_1	0.048	0.022	0.024	0.163	0.027	0.244	0.022	0.05
A_2	0.024	0.289	0.220	0.186	0.166	0.045	0.043	0.11
A_3	0.119	-	0.024	0.070	0.333	0.111	0.065	0.11
A_4	0.024	0.333	0.232	0.023	-	0.133	0.033	0.13
A_5	0.071	0.267	0.488	0.186	0.097	0.111	0.272	0.11
A_6	0.261	-	-	0.093	0.111	-	0.098	0.03
A_7	0.024	0.089	-	0.093	0.153	0.022	0.054	0.22
A_8	0.095	-	-	0.093	0.083	0.045	0.076	0.13
A_9	0.143	-	-	-	0.014	0.089	0.054	0.08
A_{10}	-	-	-	-	-	0.022	0.065	0.03
A_{11}	0.048	-	-	0.023	-	0.133	0.011	-
A_{12}	0.048	-	-	-	-	0.045	0.054	-
A_{13}	0.048	-	-	-	0.014	-	0.097	-
A_{14}	0.071	-	-	-	-	-	0.033	-
A_{15}	-	-	-	-	-	-	0.023	-

[a]Alleles are numbered such that the largest allele is assigned the lowest number and each successive allele is two bp smaller. For D6S109, A_1=215 bp, for Am10GA, A_0=123 bp, for SB1, A_0 = 220 bp, for LR40, TXSCA1 A_1 = 241 bp, (primer set A, Table 1), CEPH A_1 = 267 bp (primer set B, Table 1), for D6S202, A_1 = 154 bp.

[b]CEPH data published for D6109 (L.P.W. Ranum et al., Am. J. Hum. Genet., 49, 31-41 (1991) and D6S202 (F. LeBorgne-Demarquoy et al., Nucl. Acids Res., 19, 6060 (1991).

2. Genetic Linkage Data

a. CEPH families. In order to establish a well-defined genetic map for the SCA1 region, newly isolated DNA markers were mapped using the CEPH reference families. Results of pairwise linkage analyses in CEPH kindreds are shown in Table 3. No recombination was observed between AM10GA and D6S89 (θ = 0.00, Z_{max} = 15.1) using a subset of the CEPH panel which defined 26 recombinants between D6S109 and D6S89. The markers D6S109 and LR40 are close to D6S89, with recombination fractions of 0.067 (Z_{max} = 71.4) and 0.04 (Z_{max} = 84.5) respectively.

Selected multipoint analyses were performed to position the newly isolated markers D6S109, LR40, D6S202 with respect to markers previously mapped using the CEPH panel. The CMAP program was used for three- and four-point linkage analyses to position D6S109 relative to D6S88 and D6S89 and to position LR40 and D6S202 relative to each other and to D6S89 and F13A. For the three-point analyses, the D6S88 - D6S89 interval was fixed based on the two-point recombination fraction in CEPH and the lod score was calculated at various recombination fractions. The order D6S88 - D6S109 - D6S89 is favored over the next most likely order by odds of 4 x 10^3 : 1 (Table 4). For the four-point analyses, both the D6S89 - D6S202 - F13A and the D6S89 - LR40 - F13A intervals were fixed based on the two-point recombination fractions; lod scores were then calculated for LR40 and D6S202 at various θ values on the respective fixed maps. The order D6S89 - LR40 - D6S202 - F13A is favored over the next most likely order in both analyses; odds in favor were 400 : 1 when the position of LR40 was varied and were 1 x 10^6 to 1 when D6S202 was varied (Table 4).

The order of AM10GA and D6S89 could not be determined using the D6S109/D6S89 CEPH recombinants. However, the order AM10GA - D6S89 - SB1 was deduced by characterization of overlapping yeast artificial chromosome clones containing these markers (see below). Furthermore, one end of this contig is present in a well characterized radiation-reduced hybrid known to contain D6S109 and other centromeric markers, indicating the order D6S109 - AM10GA - D6S89 - SB1.

Table 3.

Pairwise linkage results in CEPH

Marker Pair	$\theta_m=\theta_f$	Z_{max}	θ_m	θ_f	Z_{max}	χ^2
HLA and D6S88	0.128	26.4	0.103	0.168	26.8	1.86
D6S109	0.126	48.4	0.062	0.176	51.0	12.1*
AM10	0.608	0.0440	0.301	0.500	0.246	0.929
D6S89	0.158	43.3	0.091	0.225	46.6	15.2*
SB1	0.574	0.0190	0.299	0.500	0.400	0.381
LR40	0.213	25.5	0.116	0.306	30.0	20.8*
HZ30	0.251	21.6	0.191	0.318	23.6	8.95*
F13A	0.291	8.81	0.255	0.326	9.14	1.52
D6S88 and D6S109	0.017	48.6	0.024	0.009	48.8	0.846
AM10	0.654	0.0290	0.499	0.696	0.047	0.0820
D6S89	0.086	36.1	0.076	0.098	36.2	0.0750
SB1	0.203	1.09	0.136	0.687	1.36	1.27
LR40	0.088	31.1	0.078	0.104	31.2	0.350
HZ30	0.135	30.4	0.124	0.152	30.4	0.340
F13A	0.180	10.2	0.158	0.217	10.3	0.626
D6S109 and AM10	0.730	0.933	0.170	0.502	1.67	3.39
D6S89	0.067	71.4	0.035	0.090	72.5	5.15*
SB1	0.742	1.95	0.113	0.501	4.32	10.9*
LR40	0.109	50.6	0.050	0.152	52.9	10.5*
HZ30	0.162	36.6	0.147	0.174	36.7	0.515
F13A	0.207	14.4	0.211	0.204	14.4	0.0368
AM10 and D6S89	0.000	15.1	0.000	0.000	15.1	0.000
SB1	0.000	13.2	0.000	0.000	13.2	0.000
LR40	0.021	8.74	0.000	0.050	9.11	1.74
HZ30	0.000	13.8	0.000	0.000	13.8	0.000
F13A	0.135	3.48	0.042	0.253	4.39	4.16*

D6S89 and SB1	0.000	25.0	0.000	0.000	25.0	0.000
LR40	0.040	84.5	0.030	0.049	84.7	0.925
HZ30	0.078	76.0	0.075	0.077	76.0	0.0230
F13A	0.151	30.7	0.139	0.160	30.7	0.248
SB1 and LR40	0.033	14.4	0.022	0.044	14.5	0.350
HZ30	0.026	17.5	0.032	0.020	17.5	0.0300
F13A	0.136	4.80	0.119	0.155	4.84	0.170
LR40 and HZ30	0.079	64.8	0.092	0.050	65.0	1.09
F13A	0.131	29.1	0.121	0.140	29.2	0.189
HZ30 and F13A	0.109	38.4	0.122	0.106	38.4	0.0092

*Indicates statistically significant differences were observed in the recombination fractions when the assumption of homogeneity ($\theta_m=\theta_f$) was rejected; that is the likelihood that $\chi^2 > 3.84$ with 1 degree of freedom should occur by chance in $P < 0.05$.

Table 4.

Three and four point linkage analyses in the CEPH families

Order	Z_{max}	Relative Odds	Odds in favor
D6S109-D6S88-D6S89	90.6	$2X10^{8}$	
D6S88-D6S109-D6S89	94.2	$8X10^{11}$	$4X10^{3}$
D6S88-D6S89-D6S109	82.3	1	
LR40-D6S89-D6S202-F13A	96.1	$1X10^{34}$	
D6S89-LR40-D6S202-F13A	98.6	$4X10^{36}$	400:1
D6S89-D6S202-LR40-F13A	73.9	$8X10^{11}$	
D6S89-D6S202-F13A-LR40	62.0	1	
D6S202-D6S89-LR40-F13A	89.5	$1X10^{32}$	
D6S89-D6S202-LR40-F13A	57.5	1	
D6S89-LR40-D6S202-F13A	95.5	$1X10^{38}$	10^{6}:1
D6S89-LR40-F13A-D6S202	77.6	$1X10^{20}$	

b. SCA1 kindreds. Results of pairwise linkage analyses in SCA1 kindreds are shown in Table 5. AM10GA, D6S89, and SB1 are all closely linked to SCA1. No recombination was observed between AM10GA and SCA1; the lod score is 42.1 at a recombination fraction of 0.00. The recombination fraction between D6S89 and SCA1 is 0.004 (lod score of 67.6). The recombination fraction between SB1 and SCA1 is 0.007 (lod score of 39.5). D6S109, LR40 and D6S202 are linked to SCA1 as well, but at greater distances (recombination fractions of 0.04, 0.03, and 0.08 respectively). Based on genetic mapping in nine large kindreds, the SCA1 locus is very close to D6S89 and AM10GA, with a Z_{max}-1 support interval less than or equal to 0.02 in both cases.

Table 5.

Pairwise lod scores for SCA1 and dinucleotide repeat markers

	Recombination fraction									
	0	0.001	0.05	0.1	0.2	0.3	0.4	Z^a	θ^a	Support Interval[b]
SCA1:D6S109	-∞	22.68	33.81	32.03	25.19	16.56	7.24	33.82	0.04	0.02 to 0.09
SCA1:AM10GA	42.14	42.06	38.48	34.51	25.86	16.63	7.30	42.14	0.00	0.00 to 0.02
SCA1:D6S89	-∞	67.35	62.78	56.39	42.51	27.56	12.09	67.58	0.004	0.00 to 0.02
SCA1:SB	-∞	39.02	37.33	33.92	26.16	17.53	8.33	39.46	0.007	0.00 to 0.03
SCA1:LR40	-∞	27.80	31.77	29.73	23.61	16.11	7.77	32.08	0.03	0.001 to 0.07
SCA1:D6S202	-∞	4.41	25.80	26.47	22.12	14.77	6.51	26.61	0.08	0.04 to 0.14

[a]Z = maximum lod score, θ = recombination fraction at maximum lod score.

[b]Z_{max}-1 = support interval for θ (Cytogenet Cell Genet, 40, 356-359 (1985)).

3. Analysis of Key Recombinants

One recombination event between D6S89 and SCA1 has been confirmed in an affected individual. The patient, individual MI-2 in Figure 4, was also recombinant at SB1, although uninformative at LR40 and D6S202. He carried a disease haplotype at the HLA, D6S109 and AM10 loci, demonstrating that SCA1 is centromeric to D6S89, as indicated by the rightmost arrow in Figure 4. To eliminate the possibility of sample mix-up, the patient's DNA was reextracted from a hair sample and retyped for D6S109, D6S89, D6S202, LR40, AM10GA, and SB1. The results from the hair sample matched those from the cell line originally established from the patient's blood. The patient's medical records were carefully reexamined and it was confirmed that he did indeed have ataxia. In addition, his haplotypes were consistent with those of a sister and a daughter.

D6S109 lies centromeric to D6S89; six recombination events have been observed between D6S109 and SCA1, as shown in Figure 4. At this point, D6S109 is the centromeric marker closest to SCA1. The arrows in Figure 4 denote the maximum region common to all affected chromosomes, and therefore the maximum possible region containing the SCA1 gene, which extends from D6S89 to D6S109.

No additional marker-SCA1 recombination events have been observed between D6S89 and SB1. Markers further telomeric to SB1 show additional recombination with SCA1 -- one recombination event between SCA1 and LR40 and three recombination events between SCA1 and D6S202. These events are depicted in Figure 4 (all recombination events depicted in Figure 4 are in affected individuals).

II. Mapping and Cloning the Critical Region for the SCA1 Gene

A 2.5-Mb yeast artificial chromosome (YAC) contig was developed with the ultimate goal of defining and cloning the region likely to contain the SCA1 gene (SCA1 critical region).

A. Materials and Methods

1. Cell lines

I-7 is a human-hamster hybrid cell line which contains the short arm of chromosome 6 as its only human chromosome. See, H.Y. Zoghbi et al., Genomics, 6, 352-357 (1990). R86, R78, R72, R54 and R17 are radiation reduced hybrid cell lines retaining various portions of 6p22-p23. See, H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991). R54 retains markers known to be telomeric to D6589, such as D6S202 and F13A.

2. Generation of new DNA markers and Sequence-Tagged Sites (STSs)

DNA from a radiation reduced hybrid retaining D6S89 (R86) and DNAs from four radiation hybrids (R78, R72, R54 and R17) which do not retain D6S89 but retain markers immediately flanking D6S89 were used in comparative *Alu*-PCR to isolate region-specific DNA markers. See, D.L. Nelson et al., Proc. Natl. Acad. Sci. USA, 86, 6686-6690 (1989); and H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991). In addition, R78 was useful in eliminating markers derived from the centromeric region of 6p. H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991). *Alu*-PCR was carried out using *Alu* primers 559 and 517 individually (D.L. Nelson et al., Proc. Natl. Acad. Sci. USA, 86, 6686-6690 (1989)) as well as PDJ 34 (C. Breukel et al., Nucleic Acids Res., 18, 3097 (1990)). *Alu*-PCR fragments found to be present in R86 but absent in R78, R72, R54 and R17 were identified and were cloned into *Eco*RV-digested pBluescript IIKS+ plasmid (Stratagene, La Jolla, CA) which was modified using the T-vector protocol. See, D. Marchuk et al., Nucleic Acids Res., 19, 1154 (1990). Cloned fragments were sequenced on an Applied Biosystems, Inc. (Foster City, CA) automated sequencer to establish STSs.

3. Isolation and Characterization of YAC clones

The Washington University YAC library (B.H. Brownstein et al., Science, 244, 1348-1351 (1989)), and the CEPH YAC library (H.M. Albertsen, et al., Proc. Natl. Acad. Sci. USA, 87, 4256-4260 (1990)), were screened using a PCR-based method. See, E.D. Green et al., Proc. Natl. Acad. Sci. USA, 87, 1213-1217 (1990); and T.J. Kwiatkowski et al., Nucleic Acids Res., 18, 7191-7192 (1990). PCR amplifications were carried out in 25-50 ml final volume with 50 mM KCl, 10

mM Tris-HCl pH 8.3, 1.25 mM $MgCl_2$, 0.01% (w/v) gelatin, 250 μM of each dNTP; 1.25 units of Amplitaq polymerase (Perkin-Elmer, Norwalk, CT) and 1 μM of each primer. PCR cycle conditions are specified in Table 6.

Table 6.

STSs and YACs in 6p22-p23

Probe	Primer set	YACs[a]	Annealing temp.[b]
D6S89	cttgttcatctgccttgtgcaccta agcgactgcctaaac	B126G2, B134D5, B172B3, B214D3, C5C12, 191D8, 299B3, 379C2, 468D12, 124G2, 511H11	55°C
AM10 (D6S335)	ttaaggaagtgttcacatcaggg aattgtgcttatgtcactggg	A23C3, A183C6, A250D5, B238F12, A91D2	55°C
A250D5-L (D6S337)	aattctggagagaggatgttggt tctttttttggtag	195B5, 242C5, 475A6, 30F12	44°C
64U	catcgtgttgtgtggtgaagctc agacgctaaactcaagg	492H3, 172B5, 227B1, 261H7	50°C
D6S288	atgatccgtggtagtggcagga cctgttactgacgcc	60H7, 351B10	55°C
D6S274	ctcatctgttgaatggggatctta aatgctatgccttccg	486F9, 149H3, 42A5, 283B2, 320E12	55°C
FLB1 (D6S339)	tgcaaatccctcagttcacttgctt gactttgccatgttc	140H2, 270D3, 274D12, 401D6, 57G3, 168F1	50°C
AM12 (D6S336)	atacccatacggatttgagggca acactatcaggctaagaatg	A71B3, 228A1, 193B3, 90A12, 539C11, 53G12, 35E8	55°C
53G12-L	caaataccagcaactcaccagc ggttccttcagcatcctacattc	3G6, 82G12, 98G5, 135F6, 198C8, 330G1	58°C

[a] YACs in this study are from the CEPH and Washington University libraries. I.D. numbers identify the library source (Washington University I.D. numbers are preceded by a letter). Several YACs were identified with more than one STS; for such information, please refer to Table 2.

[b] PCR conditions were 94°C for 4 minutes followed by 35-40 cycles of 94°C denaturation for 1 minute, annealing at the specified temperature for 1 minute, and 72°C extension for 2 minutes. A final extension step of 7 minutes at 72°C was used. PCR buffer and primer concentrations are as described in the text; for the 53G12-L STS a final concentration of 2% formamide was used in the PCR reaction.

Yeast DNA-agarose blocks were prepared as described by D.C. Schwartz et al., Cell, 37, 67-75 (1984); and G.J.B. van Ommen et al. in Human Genetic Diseases-A Practical Approach; K.E. Davies, ed.; pp. 113-117; IRL Press, Oxford (1986). All the YAC clones were analyzed by pulsed-field gel electrophoresis (PFGE) to determine the insert size and to confirm that a single YAC was present in a specific colony. YAC inserts were sized by electrophoresing yeast DNA through a 1% Fastlane agarose (FMC, Rockland, ME) gel in 0.5x TAE (20 mM Tris-acetate/0.5 mM EDTA). For rapid detection of possible overlaps between YAC clones isolated at different STSs, the labelled *Alu*-PCR products of new YACs were hybridized to filters containing *Alu*-PCR products of individual YACs in the region. Most of the YAC clones were tested for chimerism using the *Alu*-PCR dot blot method described by S. Banfi et al., Nucleic Acids Res., 20, 1814 (1992). The *Alu*-PCR products from YAC clones were hybridized to a dot-blot containing the *Alu*-PCR products from monochromosomal or highly reduced hybrids representing each of the 24 different human chromosomes as previously described by S. Banfi et al., Nucleic Acids Res., 20, 1814 (1992). In addition a dot-blot containing *Alu*-PCR products from radiation reduced hybrids representing different segments of 6p was used to insure that a YAC does not contain two non-contiguous segments from 6p. Ends of YAC clones were isolated either by inverse-PCR as previously described by G. Joslyn et al., Cell, 66, 601-613 (1991) or by *Alu*-vector PCR as described by D.L. Nelson et al., Proc. Natl. Acad. Sci. USA, 88, 6157-6161 (1991). *Alu*-vector PCR was carried out using *Alu*-primers PDJ34 and SAL1, as described by C. Breukel et al., Nucleic Acids Res., 18, 3097 (1990); and the pYAC4 vector primers described by M.C. Wapenaar et al., Hum. Mol. Genet., 2, 947-995 (1993) and analogous vectors described by G.P. Bates et al., Nature Genetics, 1, 180-187 (1992). All YAC ends were regionally mapped by hybridization to Southern blots containing *Eco*RI-digested DNAs from the YAC clones and from the hybrid cell lines: I-7, R86, and R72.

4. Cosmid library preparation from YACs

Cosmid libraries were prepared from four YAC clones; 227B1, 195B5, A250D5, and 379C2. Genomic DNA from YACs was partially digested with *Mbo*I and cloned into cosmid vector superCos 1 (Stratagene, La Jolla, CA) following the

manufacturer's recommendations. Clones containing human inserts were identified using radiolabeled sheared human DNA as a probe.

5. Long range restriction analysis

YAC plugs were digested to completion using rare-cutter restriction enzymes as described by M.C. Wapenaar et al., Hum. Mol. Genet., 2, 947-995 (1993) and analogously by G.A. Silverman et al., Proc. Natl. Acad. Sci. USA, 86, 7485-7489 (1989). Enzymes were purchased from New England Biolabs (Beverly, MA) and Boehringer Manheim Biochemicals (Indianapolis, IN) and were used as recommended by the manufacturer. All PFGE analyses were performed on a Bio-Rad CHEF apparatus under conditions that separate DNA fragments in the 50 kb to 600 kb range. The gels were stained with ethidium bromide, and either acid nicked or subjected to 200,000 mJ of UV energy in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The gels were denatured in 0.4 N NaOH and transferred to Sure Blot hybridization membrane (Oncor, Gaithersburg, MD) in either 10xSSC (1.5 M NaCl/150 mM NaCitrate) or 0.4 N NaOH according to the manufacturer's recommendations. Hybridizations of the filters were carried out using the probes listed in Table 6 and Figure 6. Also pBR322 *Bam*HI/*Pru*II fragments of 2.5 kb and 1.6 kb specific for the left (TRP/CEN) and right (URA) pYAC4 vector arms respectively, were used. Probes were radiolabelled using the random priming technique described by A.P. Feinberg et al., Anal. Biochem., 137, 266-267 (1984); repetitive sequences were blocked using sheared human placental DNA as previously described by P.G. Sealy et al., Nucleic Acids Res., 13, 1905-1922 (1985).

6. Dinucleotide repeat analysis

Primer sequences and PCR cycle conditions are presented in Table 6. Buffer conditions were the same as for *Alu*-PCR. All reaction volumes were 25 µl and contained 40 ng of genomic DNA. One primer of each pair was labelled at the 5' end with [γ-^{32}P] dATP. Four microliters of each reaction was mixed with 2 µl formamide loading buffer, denatured at 90-100°C for 3 minutes, cooled on ice and 4-6 µl was used for electrophoresis on a 4% polyacrylamide/7.65 M urea sequencing gel.

B. Results

1. Generation of sequence tagged sites in 6p22-p23 and YAC screening

Comparative analysis of the *Alu*-PCR products from the radiation hybrid, which retains D6S89 (R86) and from the four radiation hybrids deleted for D6S89 but retaining markers which flank D6S89 (R78, R72, R54 and R17) allowed the identification of three new DNA fragments that were present in R86 but absent in the other four. These three DNA fragments termed, AM10, AM12 and FLB1 were isolated and mapped using a 6p somatic cell hybrid panel and the radiation reduced hybrid panel (H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991)) to confirm their regional localization. All three mapped to 6p and to R86 confirming their close proximity to the D6S89 locus. These three *Alu*-PCR fragments were subcloned and sequenced to establish sequenced tagged sites (STSs). STSs at AM10, AM12, FLB1 and D6S89 were used to screen the Washington University and the CEPH YAC libraries (H.M. Albertsen, et al., Proc. Natl. Acad. Sci. USA, 87, 4256-4260 (1990); and B.H. Brownstein et al., Science, 244, 1348-1351 (1989)). YACs isolated at these four STSs were analyzed for overlap. Insert termini from the YACs representing contig ends were isolated, subcloned and were sequenced to establish new STSs for further YAC walking. In one case an STS was established by using a subclone from a cosmid derived from a cosmid library generated for YAC 195B5.

Recently several highly informative dinucleotide repeat markers have been identified and mapped genetically by J. Weissenbach et al., Nature, 359 794-801 (1992). As discussed above, two markers, D6S274 and D6S288 were found to map within the SCA1 critical region and were subsequently used to screen the YAC libraries. Using the STSs listed in Table 6, YAC clones were isolated.

2. Characterization of YAC clones

The sizes of the YAC inserts were determined by pulsed-field gel electrophoresis (PFGE); insert sizes ranged from 75-850 kb. Given the high frequency of insert chimerism, an *Alu*-PCR based hybridization strategy for rapid detection of chimerism, as described by S. Banfi et al., Nucleic Acids Res., 20, 1814 (1992) was used. Thirty of the YAC clones were tested using this approach and eight (27%) were found to be chimeric. Insert ends isolated from YACs determined

to be non-chimeric by the dot blot hybridization approach mapped to 6p22-p23 with the exception of the two ends from 198C8 which proved to map to other chromosomes.

Two approaches were used, inverse-PCR (G. Joslyn et al., Cell, 66, 601-613 (1991)) and *Alu*-PCR (analogous to that described by D.L. Nelson et al., Proc. Natl. Acad. Sci. USA, 86, 6686-6690 (1989)) to isolate YAC ends. In total, 34 YAC ends were isolated; inverse-PCR yielded 26 ends and *Alu*-vector PCR yielded 8 ends. To isolate the left end of the 195B5 YAC we screened a cosmid library prepared from this YAC using pYAC4 left end sequences (S.K. Bronson et al., Proc. Natl. Acad. Sci. USA, 88, 1676-1680 (1991)) as a probe. This approach was taken because inverse-PCR yielded an end which was predominantly an *Alu*-containing sequence and *Alu*-PCR failed in yielding an end. Cosmid clone A32 was found to contain the left end of 195B5 and a subclone, 64U, was used to establish an STS for further YAC library screenings.

In order to confirm the 6p22-p23 regional origin of all YAC ends or subclones, these fragments were used as probes against Southern blots containing *Eco*RI-digested DNAs from a somatic cell hybrid retaining 6p (I-7), from radiation reduced hybrids known to retain fragments of 6p (H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991)) and from the YAC clones at a particular STS.

3. Probe content mapping of YACs

In order to define the degree of overlap between the clones and to detect possible rearrangements such as internal deletions of the YACs, a probe content mapping strategy was used based on: 1) PCR analysis of all the clones using all the STSs in the region including both the ones described in Table 6, and those at highly informative dinucleotide repeats such as AM10-GA and SB1; and 2) hybridization of Southern blots containing *Eco*RI-digested DNAs from YACs in the relevant region, with densely-spaced DNA probes derived from YAC ends, cosmids subclones of YACs, or *Alu*-PCR fragments from YACs. The results of this analysis for a representative subset of the YACs (32 clones) are summarized in Table 7. Thirty-nine YAC clones form an uninterrupted YAC contig from D6S274 to 82G12-R (right end of YAC clone 82G12). Other than an internal deletion in one YAC (351B10) no other deletions were detected within the resolution of this analysis;

furthermore the extent of chimerism for some YAC clones (such as 270D12 and 140H2) was determined. The centromere-telomere orientation of the YAC contig on 6p was determined using both genetic data as well as physical mapping data. Using dinucleotide repeats analysis at D6S109, AM10GA, D6S89, and SB1 in the key individual with recombination event between D6S89 and SCA1 revealed that the recombination event occurred between AM10GA and D6S89. Given that D6S109 is centromeric to D6S89, the recombination analysis suggests that AM10GA is centromeric to D6S89. The centromere-telomere position of SB1 with respect to D6S89 could not be determined genetically.

TABLE 7.

Characterization of YACs using 6p22-p23 STSs and YAC fragments

YAC	Size (kb)	Chimerism	D6S274	60H7Lg	D6S288	64U	A25005-L	AM10-GA	AM10	168F1-R	C5C12-R	D6S89	B214D3-R	FLB1	53G12-R	401D6-R	AM12	135F6-L	53G12-L	135F6-R	83G12-R
149H3	345	N	+	+	-	-	-														
60H7	580	N	+	+	+	-	-														
351B10	330	N	+	-	+	-	-														
227B1	560	N	+	+	+	+	-														
172B5	345	Y	-	-	+	+	-														
195B5	365	N	-		-	+	+	-	-		-										
475A6	365	N				-	+	-	-		-										
242C5	340	N				-	+	+	+		-										
A250D5	250	N				-	+	+	+	-	-	-	-	-							
A23C3	530	Y				-	-	-	+	-	-	-	-	-							
A18306	120	N				-	-	-	+	-	-	-	-	-							
B238F12	390	Y				-	-	+	+	-	-	-	-	-							
A91D2	325	N				-	-	-	+	-	-	-	-	-							
191D8	650	N						-	+	+	+	+	+	-							
379C2	575	N						-	+	+	+	+	+	-							
C5C12	75	N						-	-	-	+	+	-	-							
B214D3	200	N							-		-	+	+	-							
299B3	375	N						-	-	+	+	+	+	+	+	-	-				
468D12	280	N							-	+	+	+	+	-							
168F1	400	N							-	+	+	+		+	+	-	-				
270D3	650	Y							-	-	+	-	+	+	+	-	-				
274D12	240	N							-	-	-	-	+	+	+	-	-				
140H2	440	Y							-	-	-	-	-	+	+	-	-				
57G3	400	N							-	-	-	-		+	+	-	-				
401D6	340	N							-	-	-	-		+	+	+	-				
193B3	850	Y							-	-	-	-	+	+	+	+	-				
228A1	350	Y												-	-	+	+	-	-	-	-
90A12	650	Y												-	-	+	+	-	-	-	-
35E8	400	N												-	-	+	+	-	-	-	-
53G12	370	N												-	+	+	+	+	+	-	-
135F6	400	N												-	+	+	+	+	+	-	-
82G12	380	N																+	+	+	-
																		-	+	+	+

Note. (+) = present, (-) = absent; Y/N = chimerism is/not detected. YAC ends are identified by YAC names followed by L or R for left or right.

Physical mapping, using both radiation hybrids and YACs, was carried out to resolve the centromere-telomere order of the loci. The radiation reduced hybrids R17 and R72 are known to contain markers centromeric to D6S89; these markers include D6S108 and D6S88 which map centromeric to D6S109. See, H.Y. Zoghbi et al., Genomics, 9, 713-720 (1991). R72 also retains D6S109, but a small gap in R17 was revealed as this radiation hybrid did not retain D6S109, but was positive for an end isolated from a YAC at the D6S109 locus. Analysis of the radiation reduced hybrids revealed that D6S274 and D6S288 are present in R17, R72 and R86, whereas AM10GA, D6S89, and SB1 are present only in R86 (Figure 5). Furthermore, STS content mapping with D6S260 and D6S289, two dinucleotide repeats that are telomeric to D6S288 (J. Weissenbach et al., Nature, 359 794-801 (1992)), revealed that D6S260 is present in the same YACs as D6S89 and SB1 (379C2 and 168F1), and that D6S289 is present in 57G3 and 35E8 two YACs derived using the FLB1 and AM12 STS respectively. These data, confirm that the order of the loci as well as the centromere-telomere orientation of the YAC contig presented in Figure 6 is correct.

Figure 6 shows a selected subset of YAC clones which span the entire contig from D6S274 to 82G12-R. A minimal number of 8 YACs spans this region. The positions of the STSs which were used to isolate the YACs are also shown. Based on the size of the YACs and the degree of overlap, this contig is estimated to span 2.5 Mb of genomic DNA in 6p22-p23 with D6S89 located approximately in the middle.

4. Delineating the SCA1 critical region

Genetic studies using recently identified dinucleotide repeats (AM10GA and SB1) showed that SCA1 maps centromeric to the D6S89 locus very close to AM10GA (peak load score of 42.1 at a recombination frequency of zero) in nine large SCA1 kindreds (Example 1, above). Thus D6S89 is the closest flanking marker at the telomeric end. Previously, the closest flanking marker at the centromeric end was D6S109, a dinucleotide repeat estimated to be 6.7 cM centromeric to D6S89. To identify a closer flanking marker at the centromeric end, we mapped D6S260, D6S274, D6S288 and D6S289, four dinucleotide repeat-containing markers known to map 6p22-p23 (J. Weissenbach et al., Nature, 359

794-801 (1992)). The regional mapping of these markers was done using radiation reduced hybrids and the YAC clones isolated from this region. These data revealed that D6S274 and D6S288 map centromeric to AM10GA as evident by amplification of DNA from radiation hybrids R17 and R72 which are known to be centromeric to AM10GA. Genotypical analysis of the DNAs from individuals with key recombination events between D6S109 and D6S89 as well as from affected and normal individuals (to establish chromosomal phase) from the five SCA1 kindreds (MN-SCA1, MI-SCA1, TX-SCA1, M-SCA1 and MS-SCA) was carried out. This analysis revealed no recombination between D6S288 and SCA1. A single recombination event between D6S274 and D6S288 was detected in individual MN-1 from the MN-SCA1 kindred (Figure 7); this individual was one of the six individuals identified above as having a recombination event between SCA1 and D6S109. This analysis allowed us to identify D6S274 as the closest flanking marker at the centromeric end. These data combined with that discussed above determined that the SCA1 critical region maps between D6S274 and D6S89. This candidate region (1.2 Mb) is cloned in a minimum of four overlapping and non-chimeric YACs as shown in Figure 8.

5. Long-range restriction mapping

In order to have an estimate of the size of the YAC contig in the SCA1 critical region we performed long-range restriction analysis on YACs from this region. The YACs used for this analysis included: 227B1, 60H7, 351B10, 172B5, 195B5, A250D5, 379C2, and 168F1. The following rare-cutter restriction enzymes were used: *Not*I, *Bss*HII, *Nru*I, *Mlu*I, and *Sac*II. Restriction fragments separated by PFGE and transferred onto nylon membranes, were detected by sequential hybridizations of the filter to several DNA probes which included: DNA probes specific for the left and right arm of the pYAC4 vector; insert termini for internal YAC clones; internal probes and cosmid subclones; and an *Alu*-specific probe. The position and names of all the probes used in the long-range restriction analysis is shown in Figure 8. Based on this analysis the internal deletion for YAC 351B10 was confirmed. The extent of overlap between the YAC clones was determined. The size of the critical SCA1 region was estimated to be 1.2 Mb. Internal deletions and/or other rearrangements could not be excluded for the areas where a single YAC

was analyzed by restriction enzyme analysis. These include approximately a 220 kb region within YAC 195B5 and a 335 kb region within YAC 379C2.

III. Expansion of an Unstable Trinucleotide Repeat in SCA1

A. Methods

1. Screening for trinucleotide repeats

Genomic DNA from YACs was partially digested with *Mbo*I and cloned into cosmid vector super CosI (Stratagene) following the manufacturer's protocol. Clones containing human inserts were identified by hybridization with radiolabeled human DNA and were arrayed on a gridded plate. Filter lifts of cosmid clones from YAC227B1 were screened for the presence of trinucleotide repeats by hybridization to [γ-^{32}P] end-labelled (GCT), oligonucleotide. In a parallel experiment, a mixture of 10 oligonucleotides representing the various permutations of trinucleotide repeats were end-labelled and hybridized to a Southern transfer of *Eco*RI-digested cosmids from YACs 195B5 and A250D5. Hybridizations were done in a solution of 1 M NaCl, 1% sodium dodecyl sulfate (SDS) and 10% (w/v) dextran sulphate. Filters were washed in 2xSSC (1xSSC is 0.15 M sodium chloride and 0.015 M sodium citrate), and 0.1% SDS at room temperature for 15 minutes, followed by a 15 minute wash at room temperature in a solution prewarmed to 67°C. Both strategies identified several positive clones, 22 of which were overlapping and contained the same 3.36-kb *Eco*RI fragment which hybridized to the $(GCT)_7$ probe and ultimately proved to have the CAG repeat by sequence analysis.

2. Genomic digests and Southern blots

Genomic DNAs were digested with *Taq*I (Boehringer Mannheim, Indianapolis, IN) or *Bst*NI (New England Biolabs, Beverly, MA) according to the manufacturers recommendations. Southern blotting was done following standard protocols.

3. DNA sequencing

To determine the DNA sequence in the region containing and flanking the CAG trinucleotide repeats, clone pGCT-7, containing the 3.36 kb-*Eco*RI

fragment, was subcloned. A 400-bp fragment with CAG trinucleotide repeats was generated from pGCT-7 by *Sau*3AI digestion and subcloned into the *Bam*HI site of pBluescriptKS- (Stratagene, La Jolla, CA) (clone pGCT-7.s1). In addition, pGCT-7 was digested with *Pst*I to remove 1.3 kb of DNA and recircularized for transformation (clone pGCT-7.p2). The position of the trinucleotide repeats was determined by PCR using $(GCT)_7$ oligonucleotide and one of the flanking sequencing primers as PCR primers. Initial results indicated that the CAG trinucleotide repeats were on the reverse primer strand, about 1.3 kb from the reverse primer, that is, 400 bp from the *Pst*I site. DNA sequencing was performed by di-deoxynucleotide chain-termination method using Sequenase and ΔTaq Cycle-Sequencing kit (United States Biochemical, Cleveland, OH). Both universal (-40) and reverse primers were used for clone pGCT-7.s1, while only universal (-40) primer was used for sequencing pGCT-7.p2.

4. RT-PCR and Northern analysis

Total RNA was extracted from lymphoblastoid cells using guanidinium thiocyanate followed by centrifugation in a cesium chloride gradient. Poly(A)$^+$RNA was selected using Dynabeads oligo$(dT)_{25}$ from Dynal (Great Neck, NY). First strand cDNA synthesis was carried out using MMLV reverse transcriptase (BRL, Gaithersberg, MD). RT-PCR was carried out using hot start PCR with three cycles of: 97°C for 1 minute, 59°C for 1 minute, and 72°C for 1 minute for the Pre1 and Pre2 primer set. Following that 33 cycles of 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute were carried out. For the Rep1 and Rep2 primer pair the same PCR cycling conditions were followed at lower annealing temperatures of 57°C and 55°C respectively. The RT-PCR products were analyzed on 6% Nusieve agarose gel. The northern blot containing various human tissues was purchased from Clonetech (Palo Alto, CA).

5. PCR Analysis

Fifty ng of genomic DNA was mixed with 5 pmol of each primer (CAG-a/GAG-b or Rep-1/Rep-2) in a total volume of 20 μl containing 1.5 mM $MgCl_2$, 300 μM dNTPs (1.25 mM $MgCl_2$ and 250 μM dNTPs for Rep-1/Rep-2 primers), 50 mM KCl, 10mM Tris-HCl pH 8.3, and 1 unit of Amplitaq (Perkin

Elmer, Norwalk, CT). For the CAG-a/CAG-b primer pair [α-^{32}P]dCTP was incorporated in the PCR reaction, for Rep-1/Rep-2 primer pair the Rep-1 primer was labeled at the 5' end with [γ-^{32}P]dATP. Formamide was used at a final concentration of 2% when using the Rep-1/Rep-2 primer pair. Samples, overlaid with mineral oil, were denatured at 94°C for 4 minutes followed by 30 cycles of denaturation (94°C, 1 minute), annealing (55°C, 1 minute), and extension (72°C, 2 minutes). Six microliters (µl) of each PCR reaction was mixed with 4 µl formamide loading buffer, denatured at 90°C for 2 minutes, and electrophoresed through a 6% polyacrylamide/7.65 M urea DNA sequencing gel. Allele sizes were determined by comparing migration relative to an M13 sequencing ladder.

B. Results

1. Cloning of the CAG repeat region in SCA1

As discussed above, in efforts to clone the SCA1 gene, key recombination events were analyzed using several dinucleotide repeat polymorphisms mapping to 6p22-p23 to identify the minimal region likely to contain the SCA1 gene. This analysis revealed that there were no recombination events between SCA1 and the centromeric marker D6S288 in five large kindreds or between SCA1 and the telomeric marker AM10GA in nine large kindreds. A single recombination event was detected between D6S274 and D6S288 identifying the closest flanking marker at the centromeric end to be D6S274. At the telomeric end, a single recombination event was detected between AM10GA and D6S89 and identified the latter as the flanking marker. A yeast artificial chromosome (YAC) contig extending from D6S274 to D6S89 and spanning the entire SCA1 candidate region was developed. A subset of the YAC clones encompassing this region is shown in Figure 9. Long-range restriction analysis determined the size of the SCA1 candidate region to be approximately 1.2 Mb. Cosmid libraries were constructed from YACs 227B1, 195B5, A250D5, and 379C2. Arrays of cosmid clones containing human inserts were hybridized with an oligonucleotide consisting of tandemly repeated CAG, as well as with oligonucleotides containing other trinucleotide repeats. Several hybridizing cosmid clones were identified, 23 of which were positive for the CAG repeat and mapped to the region between D6S288

and AM10GA (Figure 9). All 22 of these clones shared a common 3.36-kb *Eco*RI fragment that specifically hybridized to the CAG repeat.

2. Variability of the CAG Repeat Using Southern Analysis

To test the genetic stability of this repeat in SCA1, we used Southern blotting analysis to examine families with juvenile onset SCA1. A two-generation reduced pedigree from the TX-SCA1 family is shown in Figure 10a. Paternal transmission of SCA1 with an expansion of a *Taq*I fragment was noted. A 2830-bp fragment was detected in DNA from the unaffected spouse and on the normal chromosome from SCA1 patients, whereas a 2930-bp fragment was found in DNA from the affected father (onset at 25 years) and a 3000-bp fragment was detected in DNA from his affected child with an onset at 4 years. In a second SCA1 kindred, family MN-SCA1 (Figure 10b), two offspring inherited SCA1 from their father and differed in their age at onset (25 years and 9 years). These individuals also differ in the size of the amplified *Taq*I fragment they inherited from their affected father, 2900-bp and 2970-bp, respectively.

Enlargement of the $(CAG)_n$-containing fragment on SCA1 chromosomes from the same TX-SCA1 juvenile onset family was also demonstrated by Southern analysis following *Bst*NI digestion. The *Bst*NI fragment is 530-bp on normal chromosomes, is 610-bp in the SCA1 affected father, and is 680-bp in the affected juvenile onset offspring (Figure 10c). In each of these families, nonpaternity was excluded by genotypic analysis with a large number (greater than 10) of dinucleotide repeat markers. In addition, the size of the $(CAG)_n$-containing *Taq*I fragment in DNA from 30 unaffected spouses was compared to the sizes of the repeat containing TaqI fragment in DNA from 62 individuals affected with late-onset SCA1. The affected individuals are from five different SCA1 families: LA-SCA1, MI-SCA1, MN-SCA1, MS-SCA1, and TX-SCA1. In all 30 unaffected spouses fragment sizes were approximately 2830-bp and no expansions or reductions were detected with transmission to offspring. In contrast, DNA from 58 of the 62 SCA1 affected individuals contained detectably expanded *Taq*I fragments ranging in size from 2860-bp to 3000-bp in addition to the 2830-bp fragment. The DNAs from the remaining four individuals were found to have an expansion when analyzed by polymerase chain reaction (PCR). The expanded fragment always

segregated with disease, and in some cases the fragment expanded further in successive generations. In the juvenile cases the expanded restriction fragment was larger than that in the affected parent (uniformly the father in the cases analyzed) supporting the conclusion that a DNA sequence expansion is the mutational basis of SCA1.

3. Genomic DNA analysis of repeat regions

To identify the region involved in the DNA expansion, a 500-bp $(CAG)_n$-containing subclone of the 3.36-kb *Eco*RI fragment was sequenced, as was the entire 3.36-kb fragment (Figure 1). This normal allele demonstrated 30 CAG repeat units. In two of the repeat units (position 13 and 15) a T was present instead of a G.

The expansion of the trinucleotide repeat was observed in all affected individuals examined by PCR from five different kindreds representing at least two ethnic backgrounds, American Black and Caucasian. Genotypic analysis using DNA markers that are very closely linked to SCA1 (D6S274, D6S288, AM10GA, D6S89 and SB1) revealed that there are four haplotypes segregating with disease among the five families analyzed.

4. The trinucleotide repeat is transcribed

To test whether the CAG repeat lies within a gene, reverse transcription-PCR (RT-PCR) was performed using primers immediately flanking the repeat (Rep1 and Rep2) as well as primers which amplify a sequence immediately adjacent to the repeat (Pre1 and Pre2). The RT-PCR analysis confirms that the CAG repeat is present in mRNA from lymphoblasts. Furthermore, northern blot analysis of human poly(A)$^+$RNA from various tissues, using a 1.1 kb subclone (C208-1.1) from the 3.36-kb *Eco*RI fragment as a probe, identified a 10 kb transcript which is expressed in brain, skeletal muscle, placenta and to a lesser extent in kidney, lung and heart. The expression of this transcript is considerable in skeletal muscle. When the 3.36-kb *Eco*RI fragment was used as a probe on the northern blot the same size transcript was detected.

5. PCR analysis of the CAG repeat

To confirm that the CAG repeats were involved in the observed length variation, we analyzed the size of PCR-amplified fragments in 45 unaffected spouses and 31 SCA1 affected individuals using synthetic oligonucleotides that flank the CAG repeat. One pair of primers (CAG-a/CAG-b) was located within 9-bp of the repeats and identified length variation indicating that the CAG repeats are the basis of the variation.

Normal individuals displayed 11 alleles ranging from 25 to 36 repeat units (Table 8). Heterozygosity in normal individuals was 84%. Examination of this sequence in 31 individuals affected with SCA1 demonstrated that each was a heterozygote with one allele within the size range seen in the normal individuals and a second expanded allele within a range of 43 to 81 repeat units (Figure 11). Late onset SCA1 individuals showed at least 43 repeats, while 59-81 units were found in the juvenile cases. Figure 12 depicts correlation between the age-at-onset and the number of the repeat units. A linear correlation coefficient (r) of -0.845 was obtained indicating that 71.4% (r^2) of the variation in the age-at-onset can be accounted for by the number of $(CAG)_n$ repeat units. The largest trinucleotide repeat expansion was noted in SCA1 patients with juvenile onset who typically had a more rapid course. It is of interest that all of these patients were offspring of affected males, which is reminiscent of Huntington disease where there is preponderance of male transmission in juvenile cases.

Sequence analysis of the fragment containing the CAG repeat indicated that there are several extended open reading frames. Translation of the repeat in one of these frames (389-bp) would encode polyglutamine.

Table 8.
Comparison of the number of CAG repeat units on normal and SCA1 chromosomes

Number of Repeats	Normal Chromosomes		SCA1 Chromosomes	
	Number	Frequency	Number	Frequency
≥ 60	0	0	4	0.13
50 - 59	0	0	17	0.55
43 - 49	0	0	10	0.32
37 - 42	0	0	0	0
35 - 36	1	0.01	0	0
30 - 34	49	0.55	0	0
≤ 29	40	0.44	0	0
TOTAL	90	1.00	31	1.00

IV. Isolation of SCA1 cDNA

A. Methods

1. Screening of cDNA libraries.

Three cDNA libraries were screened: a human fetal brain library from Stratagene (La Jolla, CA), a human fetal brain library constructed in λ-Zap II with the inserts cloned into the *Not*1 restriction site (provided by Dr. Cheng Chi Lee at Baylor College of Medicine), and an adult cerebellar cDNA library from Clonetech (Palo Alto, CA). The libraries were plated on 150 cm plates at a density

of 50,000 pfu per plate using bacterial strain LE392 (ATCC number 33572). Hybond-N filters (Amersham, Arlington Heights, IL) were used to carry out plaque lifts. The fragments used as probes in the first screening included a mixture of two polymerase chain reaction (PCR) products obtained by using the primers Rep1 and Rep2 (Figure 3) immediately flanking the repeat and the primers Pre1 and Pre2 (Figure 3) which amplify a sequence immediately adjacent to the repeat, and a 1.1 kb subclone of the 3.36-kb *Eco*RI fragment (Figure 1). The 1.1 kb fragment (C208-1.1) is located 540 bp 3′ to the CAG repeat. A 9-kb *Eco*RI genomic fragment derived from the same cosmids containing the CAG repeat was also used in this screening. Subsequent rounds of screening were carried out on the same libraries using as probes cDNA clones 31-5, 3J, 3c7-2 and 3c7 (Figure 13). Genomic and cDNA probes were labeled using the random priming technique described in A.P. Feinberg et al., Anal. Biochem., 137, 266-267 (1984). Repetitive sequences were blocked as described in P.G. Sealy et al., Nucl. Acids Res., 13, 1905-1922 (1985). Briefly, the probes were reassociated with a large excess of shear human placental DNA. The nonrepetitive regions remained single-stranded and no separation of the single-stranded fragments from the reassociated fragments was necessary in order to allow the signal from low copy number components to be detected in subsequent transfer hybridizations. Hybridization of the filters was then carried out following standard protocols as described in H.Y. Zoghbi, et al., Am. J. Hum. Genet., 42, 877-883 (1988).

2. DNA sequencing and sequence analysis.

Shotgun libraries were constructed in M13 as described in A.T. Bankier, et al., Meth. Enzymol., 155, 55-93 (1987) for each of the following cDNA clones: 8-8, 31-5, 3c5, 3c7-1, 3J, 3c7-2, 3c7 (Figure 13). Twenty to thirty M13 subclones were sequenced for each cDNA clone using an Applied Biosystem, ABI 370A, automated fluorescent sequencer, as described in R. Gibbs, et al., Proc. Natl. Acad. Sci. U.S.A., 86, 1919-1923 (1989). Some cDNA clones (8-9b, 8-9a, AX1, B21, B11, 3c28) were partially sequenced manually using a Sequenase sequencing kit (USB, Cleveland, OH) on double-stranded templates, according to the manufacturer's recommendations. The sequence coverage in terms of numbers of cDNA/genomic clones analyzed was 3-4X in the coding and 5′UTR and 2X in the

3'UTR. All RT-PCR, 5'-RACE-PCR and inverse-PCR products were sequenced manually after subcloning into *SmaI*-digested pBluescript SK- plasmid (Stratagene, La Jolla, CA) modified using the T-vector protocol as described in D. Marchuk et al., Nucl. Acids Res., 19, 1154 (1990). Use of this protocol facilitates cloning. Briefly, *Taq* polymerase ordinarily causes a template-independent addition of adenosine at the 3' end of the PCR product, making blunt end ligations difficult. In the T-vector protocol, a thymidine is added to the 3' end of a digested plasmid. The result is a one-base sticky end complementary to the 3' adenosine in the PCR product, which greatly increases cloning efficiency.

Data base searches were carried out using the GCG software package (Genetics Computer Group, Madison, WI) and the BLAST network service from the National Center for Biotechnology Information (S.F. Altschul, et al., J. Mol. Biol., 215, 403-410 (1990)). The sequence of the SCA1 transcript has been deposited in Genbank, accession number X79204.

3. Northern blot, RT-PCR and genomic PCR analyses.

The northern blot of poly-$(A)^+$ RNA from various human tissues and the poly-$(A)^+$ RNA from adult human cerebellum were purchased from Clonetech (Palo Alto, CA). Poly-$(A)^+$ RNA from human lymphoblastoid cells was prepared by first extracting total RNA using guanidinium thiocyanate, followed by centrifugation in a cesium chloride gradient (P. Chomczynski et al., Anal. Biochem., 162, 156-159 (1987)). Poly-$(A)^+$ RNA was selected using Dynabeads oligo $(dT)_{25}$ from Dynal (Great Neck, NY). First strand randomly primed cDNA synthesis was carried out using MMLV (murine maloney leukemia virus) reverse transcriptase (BRL, Gaithersberg, MD). This was conducted in a 20 µl reaction mixture containing 3 µg RNA, first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM Mg Cl_2), (BRL, Gaithersberg, MD), 10 mM dithiothreitol (BRL, Gaithersberg, MD), 1 µM 3' end primer, 0.5 units RNasin (Promega, Madison, WI), 5.0 units MMLV reverse transcriptase (BRL, Gaithersberg, MD), 250 µM each deoxynucleotide triphospate: dGTP, dATP, dCTP, dTTP. The mixture was incubated for 20 minutes at 37°C then put on ice. A 10 µl aliquot was used for the PCR reaction. First strand randomly primed cDNA from human brain, liver and adrenal were provided by Dr. G. Borsani (Baylor College of Medicine).

RT-PCR for detection of alternative splicing was carried out with primers 9b and 5R and with primers 5F and 5R (Figure 15) under the following conditions: an initial denaturation step at 94°C for 5' followed by 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 2 minutes. The reaction mixture contained 10 µl cDNA, PCR buffer (50 mM KCL, 10 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$), 1 µM of the relevant 3' primer (primer 5R), 2% formamide and 1.25 units Amplitaq enzyme (Perkin Elmer, Norwalk, CT).

RT-PCR on lymphoblastoid cell lines with primers Rep1 and Rep2 for detection of expression of SCA1 mRNA was carried out using "hot start" PCR with three cycles of: 97°C for 1 minute, 57°C for 1 minute and 72°C for 1 minute. Following that 33 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute were carried out. Twenty microliters of the PCR reactions was then resolved on a 2% agarose gel (2 g Ultrapure agarose (BRL, Gaithersberg, MD) in 40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and blotted onto Sureblot membrane (Oncor, Gaithersburg, MD). The filter was hybridized with a $(GCT)_7$ oligonucleotide end-labeled with γ-^{32}P-ATP. Hybridizations were done in a solution of 1 M NaCl, 1% sodium dodecyl sulfate (SDS) (Sigma Chemical Company, St. Louis, MO) and 10% (w/v) dextran sulphate (Sigma Chemical Company, St. Louis, MO). Filters were washed in 2 x SSC (1 x SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), and 0.1% SDS at room temperature for 15 minutes, followed by a 15 minute wash at room temperature in a solution prewarmed to 67°C.

B. Results

Two human fetal brain cDNA libraries were screened using as probes various DNA fragments from the cosmid clone shown to contain the CAG repeat. Five cDNA clones were identified; these included clone 31-5 containing the CAG repeat, and clone 3J which was found not to overlap with 31-5 (Figure 13). Northern blot analysis revealed that clones 31-5 and 3J identified the same 11-kb transcript detectable in all tissues examined (Figure 14). Accordingly, the same two human fetal brain cDNA libraries and a human adult cerebellar cDNA library were used for several rounds of screening in order to obtain the full length transcript. As a result, 22 cDNA clones were isolated and characterized by sequence and PCR analyses to assemble a contig spanning the SCA1 transcript. Twelve of the phage

clones spanning the cDNA contig are shown in Figure 13. These clones were sequenced allowing the assembly of the entire sequence of the SCA1 cDNA which spans 10,660 bp (Figure 15).

Sequence analysis revealed a coding region of 2448 bp starting with a putative ATG initiator codon at base 936 located within a nucleotide sequence that fulfills Kozak's criteria for an initiation codon (M. Kozak, J. Cell. Biol., 108, 229-241 (1989)). An in-frame stop codon is present 57 bp upstream of that ATG in three independent cDNA clones as well as in genomic DNA. Furthermore, both the ATG at the beginning of the coding region and the upstream stop codon have been found in the murine homologue of SCA1 in the murine fetal brain library (Stratagene, La Jolla, CA). The SCA1 gene therefore encodes a polypeptide of about 816 amino acids, with an expected size of 87 kD, designated ataxin-1. However, one cannot exclude the possibility that the coding region begins at any of the other ATGs, located downstream of the first methionine, which would result in a smaller protein.

The CAG repeat is located within the coding region 588 bp from the first methionine and encodes a polyglutamine tract. The open reading frame ends with a TAG stop codon at base 3384. Therefore, this transcript has a 5′ untranslated region (5′UTR) of 935 bp and a 3′ untranslated region (3′UTR) of 7277 bp. The transcript ends with a tail of 57 adenosine residues; a polyadenylation signal, AATAAA, is found 23 nucleotides upstream of the poly(A) tail. Homology searches using both the DNA sequence of the coding region and the predicted protein sequence (lacking the CAG repeat and the polyglutamine tract, respectively) revealed no significant homology with other known proteins in the data base. Analysis of the sequence of ataxin-1 failed to reveal the presence of any strong phosphorylation sites as well as any specific motifs such as DNA binding or RNA binding domains. The putative secondary structure of this protein is compatible with that of a soluble protein as no hydrophobic domains were identified. A DNA sequence data base search revealed an identity between 380 bp in the 3′UTR of the SCA1 transcript and an expressed sequence tag (EST04379) isolated from a human fetal brain cDNA library (M.D. Adams, M.D. et al., Nature Genet., 4, 256-267 (1993)).

V. Organization of the SCA1 Transcript: Evidence for Alternative Splicing in the 5'UTR

A. Methods

1. 5'-RACE-PCR

First strand cDNA was prepared from 1 mg of poly-$(A)^+$ RNA from human adult cerebellum (Clonetech, Palo Alto, CA) using the primer 5R (Figure 15) as described in Example IV. 5'-RACE-PCR was carried out as described in M.A. Frohman in PCR Protocols. A Guide to Methods and Applications; M.A. Innis, et al., Eds.; Academic Press: San Diego (1990) using SCA1 primers 5a and X4-1 (Table 9) as specific primers. The product was then electrophoresed through a 1.2% agarose gel, blotted onto SureBlot hybridization membrane (Oncor, Gaithersburg, MD) as described in Example II above, and then, to test the specificity of the product, hybridized to a SCA1 specific probe represented by a PCR product spanning 118 bp between primer 9b in exon 1 and primer X3-1 (Table 9) in exon 3.

Table 9.

Primer sequences for inverse-PCR

Exon	Primer 1	Primer 2
2	**X2-1** (181-164) GTAGTAGTTTTTGTGAGG	**X2-2** (185-203) CACCAAGCTCCCTGATGGA
3	**X3-1** (246-229) GCTTGAATGGACCACCCT	**X3-2** (277-296) ATCTCCTCCTCCACTGCCAC
4	**X4-1** (347-329) AGACTCTTTCACTATGCTC	**X4-4** (407-425) TTCAGCCTGCACGGATGGT
5	**5a** (482-463) TGGCAGTGGAGAATCTCAGT	**5-2** (519-538) TGCTGCAAGGAACTGATAGC
6	**10a** (598-580) AATGGTCTAATTTCTTTGG	**10b** (607-625) GAGAAAGAAATCGACGTGC
7	**6-1** (714-695) ACAGGCTCTGGAGGGCTCCT	**X5-2** (723-742) TCCATGGTGAAGTATAGGCT
9	**9-1** (2919-2900) AGCAGGATGACCAGCCCTGT	**9-2** (2939-2957) GCTCTTTGATTTGCCGTGT
All primers are read in the 5' to the 3' direction. Numbers in parenthesis represent the coordinates of each primer within the SCA1 cDNA sequence (Figure 15).		

B. Results

To characterize the genomic region flanking the CAG repeat, the 3.36-kb *Eco*RI genomic fragment known to contain this repeat was completely sequenced. Alignment of this genomic sequence with the cDNA sequence allowed us to determine that the 3.36-kb *Eco*RI fragment contains a 2080-bp exon which has 160 bp of 5'UTR, the first potential initiation codon and the first 1920 bp of the coding region. The rest of the coding region lies within the next downstream exon as detected by PCR analysis on genomic DNA. The last coding exon, which maps to a 9-kb *Eco*RI fragment in genomic DNA also contains 7277 bp of 3'UTR for a total length of 7805 bp (Figure 16*a*).

Evidence for alternative splicing in the 5'UTR was initially suggested based on the hybridization pattern of the two most 5' cDNA clones, 8-8 and 8-9b (Figure 13) to Southern blots containing *Eco*RI-digested genomic DNA from total human DNA and YACs spanning the SCA1 region. At least three strongly hybridizing fragments in addition to the 3.36-kb *Eco*RI fragment were seen. As neither of the cDNA clones contains an *Eco*RI site, this result suggested the presence of several exons in the 5'UTR of the SCA1 transcript. Given these data and the unusual length of the 5'UTR, this region was characterized in more detail.

Alignment analysis of the sequence of clones 8-8 and 8-9b revealed the presence of two different 5' sequences diverging at basepair 322. This result was highly suggestive of alternative splicing. In order to test this hypothesis, reverse transcription-PCR (RT-PCR) was performed on mRNA from cerebellar tissue using the primers indicated in Figure 15. When the primers 9b (specific for 8-9b clone) and 5R (present in both clones) were used in the RT-PCR analysis three products were obtained: one of the expected size (246 bp) and at least two fragments of larger size (Figure 16*b*). The same result was obtained when RT-PCR was carried out on liver, adrenal, brain and lymphoblast cDNAs. The various RT-PCR products were cloned and sequenced. Sequence analysis of all these products and comparison with the sequence of phage clones 8-8 and 8-9b confirmed that they were the result of alternative splicing. Figure 16*a* shows the structure of all the cDNA clones which contain the 5' exons of the SCA1 gene and depicts the splice variants. Based on sequence analysis of three cDNA clones and characterization of cerebellar RT-PCR products, five exons (exons 1 through 5) were identified and

their borders in the transcript were determined. Exons 2, 3 and 4 are alternatively spliced in the clones examined and in cerebellar tissue, whereas exon 5 was present in all the cDNA clones and RT-PCR products.

Rescreening of cDNA libraries with clones 8-8 and 8-9b as probes did not yield any additional cDNA clones. To identify additional alternatively spliced exons in the 5'UTR and to confirm initial results, 5'-RACE-PCR was carried out on reverse transcribed cerebellar mRNA using primers from the 5' end of exons 5 and 4. A 218-bp product was identified and its specificity was confirmed by Southern analysis using an internal PCR product as probe. Sequence analysis of the 5'-RACE-PCR product, furthermore, confirmed the alternative splicing of two exons (2 and 3) and allowed the identification of an additional 127 bp at the 5' end of this gene (Figure 16*a*).

VI. Identification of Intron-Exon Boundaries and Determination of the Genomic Structure of SCA1

A. Methods

1. Identification of intron-exon boundaries

The boundaries of exons 2-9 were identified by inverse-PCR. To carry out inverse-PCR, YAC agarose plugs were digested to completion as described in M.C. Wapenaar, et al., Hum. Mol. Genet., 2, 947-952 (1993) using frequent-cutter restriction enzymes such as *Sau*3aI, *Taq*I, *Hae*III and *Msp*I purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and used as recommended by the manufacturer. The plugs were then digested with β agarase I (USB, Cleveland, OH) following the manufacturer's recommendations and subsequently phenol-chloroform (Boehringer Mannheim Biochemicals, Indianapolis, IN) extracted, precipitated with ethanol and resuspended in 12 ml of TE (TE: 10 mM Tris-HCl, 1 mM EDTA) pH 8. Fifty ng of DNA from each digest was then circularized according to the published protocol of J. Groden et al., Cell, 66, 589-600 (1991). Diverging PCR primers were designed within the cDNA and used on the circularized product under the amplification conditions described in J. Groden et al., Cell, 66, 589-600 (1991). PCR products were then subcloned and sequenced as described in Example II, above. Inverse-PCR identified all intron/exon boundaries except the boundary of exon 1. Accordingly, a 9-kb *Eco*RI

genomic fragment found to contain exon 1 was subcloned from a cosmid derived from YAC 227B1. (Example II). This subclone was subsequently partially sequenced to identify the boundary of exon 1.

2. Mapping of cDNA clones to the YACs and cosmids.

Southern blots containing *Eco*RI-digested DNAs from YACs spanning the SCA1 critical region as well as Southern blots containing DNAs from the YACs digested with rare-cutter enzymes (see previous section) were hybridized, using the standard protocol described in H.Y. Zoghbi et al., Am. J. Hum. Genet., 42, 877-883 (1988), to various SCA1 cDNA clones and to all the genomic fragments containing the intron-exon boundaries. Briefly, restriction fragments were separated by electrophoresis on 0.7% agarose gels, denatured and transferred to Nytran (Schliecher and Schuell, Keene, NH) filters. Probes were ^{32}P-labeled using the oligohexamer labeling method (A.P. Feinberg et al, Anal. Biochem., 132, 6-13 (1983)). After hybridization the filters were washed and autoradiography was performed, as described in Zoghbi et al., Am. J. Hum. Genet., 42, 877-883 (1988).

B. Results

Complete sequencing of the 3.36-kb *Eco*RI fragment provided the intron-exon boundaries for the 2080-bp exon containing most of the coding region (Figure 17). In order to determine the actual number of exons and to obtain all of the intron-exon boundaries, an inverse-PCR strategy was adopted using two overlapping YAC clones, 227B1 and 149H3, known not to contain any rearrangements (see Example II). A total of nine exons, seven of which are in the 5'UTR, were identified and splice junctions for exons 1 through 9 were subcloned and sequenced (Figure 17). The schematic on top of Figure 16*a* shows the nine exons and their respective sizes. In the 5' untranslated region, alternative splicing involves exons 2, 3 and 4, but not exons 5, 6 and 7 in over 5 phage cDNA clones analyzed. The putative exon 1 encompasses 157 bp and hybridizes very strongly to an *Eco*RI fragment derived from hamster genomic DNA.

To study the genomic organization of the SCA1 gene, ten cDNA clones as well as genomic fragments containing the splice junctions for all the exons were mapped by Southern analysis and localized on a long range restriction map of

four overlapping YAC clones spanning the SCA1 critical region (Figure 18). This analysis revealed that the gene spans at least 450 kb of genomic DNA and that the putative first exon maps to a genomic fragment containing a hypomethylated CpG island. Detailed restriction analysis of the intron between the two coding exons (8 and 9) revealed that this intron is approximately 4.5-kb in length. The sizes of the remaining introns were estimated from the long range restriction map and by PCR analysis and ranged from 650 bp (intron 2) to nearly 200 kb (intron 7) (Figure 18).

VII. Expression of the SCA1 mRNA in SCA1 Patients

As a first step toward understanding the mechanism by which the expansion of a trinucleotide CAG repeat leads to neurodegeneration in SCA1, the level of transcription of SCA1 from the expanded alleles in patients was investigated. RT-PCR was carried out with primers Rep1 and Rep 2 which flank the CAG repeat as described in Example V using lymphoblastoid mRNAs from SCA1 patients with repeat sizes ranging from 43 to 69. This analysis revealed that mRNA was expressed from both the normal allele and the expanded allele (Figure 19).

VIII. Cloning of portions of the SCA1 Gene into the pMAL™-2 Vector

DNA from the SCA1 gene was cloned into the pMAL™-c2 vector (New England Biolabs, Beverly, MA), which produces a chimeric protein consisting the maltose-binding protein fused to the N-terminus of the protein of interest (ataxin-1) in a linkage that can subsequently be conveniently cleaved. To obtain DNA for cloning, SCA1 DNA was amplified and isolated clone 31-5 (Figure 13) using standard PCR techniques. The manufacturer's instructions were followed in designing the appropriate oligonucleotide primers (pMAL™ vector Package Insert, 1992 New England Biolabs, revised 4/7/92). In each case an *Eco*RI linker site was designed into the 5' primer and a *Hin*dIII linker site was designed into the 3' primer to facilitate cloning. Three different amplification products were obtained. In one, DNA was isolated utilizing two 20-mer PCR primers COD and RCOD (Table 10) that hybridized to the 5' and 3' ends of the coding regions, such that the stretch of DNA being amplified contained residues presumed to encode the entire sequence of ataxin-1, beginning with Met1 and ending with Lys 817 (Figure 15). The amplified product was than cloned into the *Eco*RI/*Hin*dIII site in the polylinker region of in

pMAL™-c2 following instructions provided by the manufacturer. Two other constructs were made in the same way using PCR to isolate shorter segments of DNA. In both cases the same 3' end primer was used, but different 5' primers were employed (Table 10). One 5' primer (3COD) was designed such that the amplified product began at Met277 (the fourth methionine in the coding region), the other 5' primer (8COD) such that the amplified product began at Met548. pMAL™-c2 was transformed into competent cells containing a lacZΔM15 allele for α-complementation and cultured as recommended by the manufacturer.

Table 10.

Primers for Cloning Into pMal Vector

Primer Name	Nucleotide Sequence
COD	TGT GAA TTC ATG AAA TCC AAC CAA GAG CG
3COD	TGT GAA TTC ATG ATC CCA CAC ACG CTC AC
8COD	TGT GAA TTC ATG GTG CAG GCC CAG ATC
RCOD	TTC GAA GCT TCT ACT TGC CTA CAT TAG AC

IX. Expression of Ataxin-1, Design of Antigenic Peptides and Production of Antibodies

The fusion protein expressed by the constructs in Example VII were purified as directed by the manufacturer using affinity chromatography (pMAL™ vector Package Insert, 1992 New England Biolabs, revised 4/7/92). The purified protein was electrophoresed using 8% SDS polyacrylamide electrophoresis and electroeluted. The best expression (about 27 mg from 1 L of cells) was obtained from the shortest construct, but all constructs produced measurable levels of protein of a size consistent with their respective cloned gene product.

Antibody response in rabbits was initiated using the multiple antigenic peptide strategy of V. Mehra et al., Proc. Natl. Acad. Sci. USA, 83, 7013-7017 (1986). In addition to the three electroeluted cloned gene products described in the preceding paragraph, three synthetic peptides were used as well. The synthetic peptides used were Peptide A (amino acids 4 through 18), Peptide B

(amino acids 162 through 176) and Peptide C (amino acids 774 through 788). These peptides were chosen such that they showed little or no homology with other known short amino acid stretches in proteins and also such that they contained proline, which makes it more likely that these fragments are located on the surface of the protein, thus making it more likely that antibodies to the fragments will react with the whole protein as well.

Immunoglobulin (IgG) from rabbit blood was purified, and antibody/antigen results were analyzed using Western blots as described in Gershoni et al., Anal. Bioch., 131, 1-15 (1983). IgG from rabbits injected with the cloned gene products and the synthetic sequences were found to hybridize to their respective antigens. The anti-sera from rabbits immunized with the 8COD-RCOD gene product (i.e., the ataxin-1 fragment spanning residues 548 through 817) hybridized with a protein of the expected size in brain tissue extracts from mouse, rats, and humans. A similar size protein has also been detected using lymphoblasts. This hybridization is blocked by preincubation with the polypeptide antigen, and not blocked by unrelated antigens. In particular, antibodies raised against Peptide C are blocked by either Peptide C or the short gene product.

X. Molecular and Clinical Correlations in Spinocerebellar ataxia type 1 (SCA1)

A. Materials and Methods

1. Family Material

Members representing 87 kindreds with dominantly inherited ataxia were evaluated. Nine kindreds of diverse ethnic background (Caucasian American, African American, South African, Siberian Iakut) were already known to have SCA1 based on linkage to the HLA locus and to D6S89 on chromosome 6p. Genotypic analysis of the SCA1 CAG repeat was carried out on all nine kindreds to determine if all known SCA1 families had the same mutational mechanism involving repeat expansion. Most of the study participants were personally examined. The affected status was always confirmed by a neurologist, but the age of onset was based on historical information from the patient and/or other family members. Severity of disease was measured by the age at death minus the age of onset. Detailed characterization of the repeat variability was carried out for all nine

kindreds. To identify additional kindreds with a CAG expansion at the SCA1 locus, affected individuals from 78 newly identified families with dominantly inherited ataxia were clinically examined. Blood was collected from at least one affected individual from each of these kindreds and screened by DNA analysis for the presence of a CAG repeat size within the expanded range (≥ 42 repeats). Although there was no evidence that these 78 individuals are related, there is a chance that some of the affected patients come from the same families.

To assess the distribution of CAG repeat sizes on normal chromosomes further, the number of CAG repeats was determined for 304 normal chromosomes from unrelated individuals of various ethnic backgrounds.

2. Molecular Studies

Blood samples were used to establish lymphoblastoid cell lines by Epstein-Barr virus transformation. Genomic DNA was isolated either directly from venous blood or from lymphoblastoid cell lines. Blood samples were collected from these patients over an 8-year period, during which time 29 patients died. PCR reactions were performed using the Rep1 (TTGACCTTTACACCTGCAT) and Rep2 (CAACATGGGCAGTCTGAG) primers. Fifty nanograms of genomic DNA was mixed with 5 pmol of each primer in a total volume of 20 µl containing 1.25 mM $MgCl_2$, 250 uM dNTPs, 50 mM KCl, 2% formamide, 10 mM Tris-HCl pH 8.3 and 1 unit ampliTaq (Perkin-Elmer/Cetus). The Rep1 primer was labelled at the 5' end with [γ-^{32}P]ATP. Samples were denatured at 94°C for 4 minutes, followed by 30 cycles of denaturation (94°C, 1 minute), annealing (55°C, 1 minute) and extension (72°C, 2 minutes). Six µl of each PCR reaction was mixed with 4 µl formamide loading buffer, denatured at 90°C for 2 minutes, and electrophoresed through a 6% polyacrylamide/7.65 M urea DNA sequencing gel. Allele sizes were determined by comparing migration relative to an M13 sequencing ladder.

3. Statistical Analyses

The relationship between age of onset and CAG repeat number on both the affected and the normal chromosomes of patients was evaluated through linear regression analyses. Similarly, the relationship between repeat length and duration of disease was quantified. Ages of onset were used directly in these

analyses, but also following logarithmic and square root transformation. Although the latter transformation provided the best approximation to a normal distribution, results obtained were consistent between analyses before and after transformation. Analysis of variance was performed to detect differences among the families in the mean age of onset, after correction for the effect of the CAG repeat number on age of onset. In addition, the sex of the transmitting parent was included as a possible explanatory variable for variations in age of onset. All regression and variance analyses were carried out with the SPSS package of computer programs, versions 4.0.1.

B. Results

1. Family Studies

All affected individuals from the nine known SCA1 kindreds had an expanded trinucleotide repeat on one of their alleles. No repeat expansions were observed among eight kindreds previously shown by linkage analyses not to be SCA1. These eight kindreds were examined for the SCA1 gene expansion to confirm the linkage results.

Among the 70 other dominant ataxia families analyzed, three (4%) were found to have an expanded CAG repeat on one of the SCA1 alleles. Of all of the dominant kindreds studied, 12 of 87 (14%) have an expanded CAG repeat at the SCA1 locus. While the sample size is relatively small, and both estimates are arguably biased to exclude or select for SCA1 kindreds, expanded CAG repeat tracts within the SCA1 gene clearly account for only a small fraction of this complex group of diseases. The distribution of the CAG repeat number from normal controls and from ataxic individuals that did not have an expansion were similar (data not shown). These data argue against the involvement of the CAG repeat at the SCA1 locus in these families. However, it is still possible that some of these small families have other mutations at the SCA1 locus.

The typical clinical findings in the genetically proven SCA1 kindreds were gait and limb ataxia, dysarthria, pyramidal tract signs (spasticity, hyperreflexia, extensor plantar responses) and variable degrees of occulomotor findings which include one or more of the following: nystagmus, slow saccades, and opthalmoparesis. In the later stages of the disease course, bulbar findings consistent

with dysfunction of cranial nerves IX, X, and XII became evident. Also, dystonic posturing and involuntary movements including choreoathetosis became apparent in the later stages of the disease. Motor weakness, amyotrophy, and mild sensory deficits manifested as propioceptive loss were also detected. Although ataxia, dysarthria and cranial nerve dysfunction were consistently present in every SCA1 affected individual, considerable intrafamilial variability was noted with regard to all of the other clinical features. Juvenile onset (≤ 18 years) was observed in four kindreds. Of interest is the finding that juvenile onset cases typically inherited the disease gene from an affected father. Several of the kindreds that did not have an expanded SCA1 CAG repeat, displayed the same clinical findings as those observed in SCA1 kindreds confirming the inherent difficulty in clinically classifying this group of disorders. While it is possible that some of these kindreds have other mutations at the SCA1 locus, the disease locus (loci) for eight of these families has also been excluded from the SCA1 region by linkage analyses.

2. Repeat Analysis on Normal and SCA1 Chromosomes

Figure 20 shows the size distribution of the CAG repeats on 304 chromosomes from unaffected control individuals who are at risk for ataxia, and 113 expanded alleles from individuals affected with the disease. The normal alleles range in size from 19 to 36 CAG repeat units. Over 95% of the normal alleles contain from 25 to 33 CAG repeat units, the majority (65%) of which contain 28 to 30 repeats. The mean repeat size on normal chromosomes for the African Americans, Caucasian, and South African populations are very similar with 29.1, 29.8, and 29.4 CAG repeat units, respectively. Combined heterozygostiy for the CAG repeat at the SCA1 locus was 0.809 for the populations examined, giving an overall polymorphism information content (P.I.C.) value of 0.787. No change in CAG repeat length was observed for 135 meioses of SCA1 alleles containing CAG repeat tracts within the normal range, i.e., all were inherited in a Mendelian fashion. In contrast, 41 of the 62 meioses involving expanded SCA1 alleles changed in repeat size. The rate of repeat instability for female meioses is 60% while the instability observed for males was 82%.

The number of CAG repeats found on SCA1 chromosomes from 113 affected individuals was always greater than the number of repeats on normal chromosomes, ranging from 42 to 81 with a means of 52.6 (Figure 20).

All patents, patent documents, and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule containing a CAG repeat region of an isolated autosomal dominant spinocerebellar ataxia type 1 (SCA1) gene, said gene located within the short arm of chromosome 6.

2. The nucleic acid molecule of claim 1 corresponding to the entire SCA1 gene.

3. The nucleic acid molecule of claim 1 wherein the SCA1 gene encodes ataxin-1.

4. The nucleic acid molecule of claim 3 of about 2.4-11 kb in length containing the coding region of the SCA1 gene.

5. The nucleic acid molecule of claim 1 wherein the CAG repeat region is represented by $(CAG)_n$ and n = 2-36.

6. The nucleic acid molecule of claim 5 wherein n = 19-36.

7. The nucleic acid molecule of claim 1 wherein the CAG repeat region is represented by $(CAG)_n$ and $n > 36$.

8. The nucleic acid molecule of claim 7 wherein $n \geq 43$.

9. The nucleic acid molecule of claim 1 wherein the molecule is a single-stranded polynucleotide.

10. The nucleic acid molecule of claim 9 wherein the single stranded polynucleotide is cDNA.

11. The nucleic acid molecule of claim 9 wherein the single stranded polynucleotide is mRNA.

12. The nucleic acid molecule of claim 1 wherein the nucleic acid is genomic DNA.

13. An isolated oligonucleotide that hybridizes to a nucleic acid molecule containing a CAG repeat region of an isolated SCA1 gene; said oligonucleotide having at least about 11 nucleotides.

14. The isolated oligonucleotide of claim 13 having at least about 16 nucleotides.

15. The isolated oligonucleotide of claim 14 having no more than about 35 nucleotides.

16. The isolated oligonucleotide of claim 13 that produces a primed product of about 70-350 base pairs.

17. The isolated oligonucleotide of claim 16 that produces a primed product of about 100-300 base pairs.

18. The isolated oligonucleotide of claim 13 that hybridizes to the nucleic acid molecule within about 150 nucleotides on either side of the CAG repeat region.

19. The isolated oligonucleotide of claim 18 that hybridizes to the nucleic acid molecule directly adjacent to the $(CAG)_n$ region.

20. The isolated oligonucleotide of claim 13 having at least about 100 nucleotides.

21. The isolated oligonucleotide of claim 20 having at least about 200 nucleotides.

22. The isolated oligonucleotide of claim 13 comprising a nucleotide sequence selected from the group consisting of CCGGAGCCCTGCTGAGGT (CAG-a), CCAGACGCCGGGACAC (CAG-b), AACTGGAAATGTGGACGTAC (Rep-1), CAACATGGGCAGTCTGAG (Rep-2),

CCACCACTCCATCCCAGC (GCT-435), TGCTGGGCTGGTGGGGGG (GCT-214), CTCTCGGCTTTCTTGGTG (Pre-1), and GTACGTCCACATTTCCAGTT (Pre-2).

23. A method for detecting the presence of a DNA molecule containing a CAG repeat region of the SCA1 gene comprising:
    (a) digesting genomic DNA with a restriction endonuclease to obtain DNA fragments;
    (b) probing said DNA fragments under hybridizing conditions with a detectably labeled gene probe, which hybridizes to a nucleic acid molecule containing a CAG repeat region of an isolated SCA1 gene having at least about 11 nucleotides;
    (c) detecting probe DNA which has hybridized to said DNA fragments; and
    (d) analyzing the DNA fragments for a CAG repeat region characteristic of the normal or affected forms of the SCA1 gene.

24. The method of claim 23 wherein the step of analyzing comprises analyzing for a $(CAG)_n$ region wherein $n > 36$.

25. The method of claim 24 wherein the step of analyzing comprises analyzing for a $(CAG)_n$ region wherein $n \geq 43$.

26. The method of claim 23 wherein the detectably labelled DNA sequence comprises a portion of an *Eco*RI fragment of the SCA1 gene.

27. The method claim 26 wherein the *Eco*RI fragment comprises about 3360 base pairs.

28. A method for detecting the presence of a DNA molecule located within an affected allele of the SCA1 gene comprising:
    (a) treating separate complementary strands of a DNA molecule containing a CAG repeat region of the SCA1 gene with a molar excess of two oligonucleotide primers;
    (b) extending the primers to form complementary primer extension products which act as templates for synthesizing the desired molecule containing the CAG repeat region;
    (c) detecting the molecule so amplified; and
    (d) analyzing the amplified molecule for a CAG repeat region characteristic of the SCA1 disorder.

29. The method of claim 28 wherein the step of analyzing comprises analyzing for a $(CAG)_n$ region wherein $n > 36$.

30. The method of claim 29 wherein the step of analyzing comprises analyzing for a $(CAG)_n$ region wherein $n \geq 43$.

31. A protein encoded by the SCA1 gene having therein a glutamine repeat region.

32. The protein of claim 31 having a molecular weight of about 20-90 kD.

33. The protein of claim 31 having the amino acid sequence shown in Figure 15.

34. An antibody to a protein encoded by DNA containing a CAG repeat region of the SCA1 gene.

35. A method for detecting the SCA1 disorder comprising:
    (a) contacting an antibody to a protein encoded by the SCA1 gene with a biological sample containing antigenic protein to form an antibody-antigen complex;
    (b) isolating the antibody-antigen complex; and

(c) sequencing the antigen portion of the antibody-antigen complex using amino acid sequencing techniques.

FIGURE 1

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   1 TTTTGAAACT TGCAGAGAAC AGGATTATTT CTGGCGGCCT CTGCTGAGTT GGCGTGTGTG
  61 TGTGTGTTTG TGTGTGTGTG TATTAGGGAG AGGAAATCGT AGGTCCAGTG TGGACCCAGA
 121 GCTAAGGGGA ATCTTGGAGA GTAGTGGCTC TGGCAGATGA GGATTCAGAA ATCGAGTGCA
 181 AGGACTGTTC TGGACTTTCA CTGCTAACCT GCTTTTTCTC AGTGCCTGGC TCTGAGGGCA
 241 GGGTCCAGCT GGTGTCATGC TCTCCAAGGG CTTCATTTTA TGTTCCAGCC AGGCAAAGGA
 301 GAGGTGAGAA ATGGAACCAA CATTTCTGAA AAGGAAATTT AAGAACTGCA TCATCTGCCC
 361 TTGAAGAAGA AAAGGAGAAA AAAAAACAGG AGAGAGGGTA TTGAGAACAT CTTAGGGGAG
 421 TTGTTAACTC CATTAAAAAA TATATGTGTT ACAGTGTTCA CTTGCCCAGT GTCTTCATAA
 481 TCTTCCTTTA TAATGTGCAG CTGCCACGGC TAGTGTTTTT GTTTTTGTTG TTGTTGTTTT
 541 GTTTCGTTTT TGGAGACAGA GTGTCGCTCT GTTGCCCAGG CTGGAGTACA ATGGTGCAAT
 601 CTCGGCTCAC TGCAACCTCT GCCTCCTGGG TTCAAGCAAT TCTCCTGCCT CAGCCTCTCA
 661 AGTAGCTGGG ACTACAGCCG TGTGCCAGCT AATGTTACAC CAGGCTAAAT TTGTTTTTTA
 721 TTTTTTATTT TTGGTAGAGA CGGGGTTTCA CCATGTTAGC CAGGATGGTC TTAATCTCCT
 781 GACCTCGTGA TCTGCCTGCC TCGGCCTCCC AAAGTGTTGG CTAGTGTTTT CTCTGCTTCA
 841 GTGCTTGGGG TATGATTGGG TTATGGGAGT TCACACCGAG TCCAGGGCCT AGTCTTAATC
 901 TTGCCAAAGA TGTTCTTTCC CCGGTGCTCA TGTTCTGATG TCCTTTCCCT CCTTCCCTTT
 961 CTCCTCCCTT TCCTTTTCCC TTTGTCACTG CCCTCTTCCC TTTCCCAGCA TCCAGAGCTG
1021 CTGTTGGCGG ATTGTACCCA CGGGGAGATG ATTCCTCATG AAGAGCCTGG ATCCCCTACA
1081 GAAATCAAAT GTGACTTTCC GTTTATCAGA CTAAAATCAG AGCCATCCAG AACAGTGAAA
1141 CAGTCACCGT GGAGGGGGGA CGGCGAAAAA TGAAATCCAA CCAAGAGCGG AGCAACGAAT
1201 GCCTGCCTCC CAAGAAGCGC GAGATCCCCG CCACCAGCCG GTCCTCGGAG GAGAAGGCCC
1261 CTACCCTGAC CCAGCGACAA CCACCGGGTG GAGGGCACAG CATTGGCTCC CGGGCAACCC
1321 TGGTGGCCGG GGCCACGGGG GCGGGAGGCA TGGGCCGGCA GGGACCTCGG TGGAGCTTGG
1381 TTTACAACAG GGAATAGGTT TACACAAAGC ATTGTCCACA GGGCTGGACT ACTCCCCGCC
1441 CAGCGCTCCC AGGTCTGTCC CCGTGGCCAC CACGCTGCCT GCCGCGTACG CCACCCCGCA
1501 GCCAGGGACC CCGGTGTCCC CCGTGCAGTA CGCTCACCTG CCGCACACCT TCCAGTTCAT
1561 TGGGTCCTCC CAATACAGTG GAACCTATGC CAGCTTCATC CCATCACAGC TGATCCCCCC
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1741 CCGGGACACA AGGCTGAGCA GCAGCAGCAG CAGCAGCAGC AGCAGCAGCA GCAGCATCAG
1801 CATCAGCAGC AGCAGCAGCA GCAGCAGCAG CAGCAGCAGC AGCAGCAGCA CCTCAGCAGG
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1921 ATTTCCAGTT CTCCGCAGAA CACCGGCCGC ACCGCCTCTC CTCCGGCCAT CCCCGTCCAC
1981 CTCCACCCCC ACCAGACGAT GATCCCACAC ACGCTCACCC TGGGGCCCCC CTCCCAGGTC
2041 GTCATGCAAT ACGCCGACTC CGGCAGCCAC TTTGTCCCTC GGGAGGCCAC CAAGAAAGCC
2101 GAGAGCAGCC GGCTGCAGCA GGCCATCCAG GCCAAGGAGG TCCTGAACGG TGAGATGGAG
2161 AAGAGCCGGC GGTACGGGGC CCCGTCCTCA GCCGACCTGG GCCTGGGCAA GGCAGGCGGC
2221 AAGTCGGTTC CTCACCCGTA CGAGTCCAGG CACGTGGTGG TCCACCCGAG CCCCTCAGAC
2281 TACAGCAGTC GTGATCCTTC GGGGGTCCGG GCCTCTGTGA TGGTCCTGCC CAACAGCAAC
2341 ACGCCCGCAG CTGACCTGGA GGTGCAACAG GCCACTCATC GTGAAGCCTC CCCTTCTACC
2401 CTCAACGACA AAAGTGGCCT GCATTTAGGG AAGCCTGGCC ACCGGTCCTA CGCGCTCTCA
2461 CCCCACACGG TCATTCAGAC CACACACAGT GCTTCAGAGC CACTCCCGGT GGACTGCCAG
2521 CCACGGCCTT CTACGCAGGG ACTCAACCCC CTGTCATCGG CTACCTGAGC GGCCAGCAGC
2581 AAGCAATCAC CTACGCCGGC AGCCTGCCCC AGCACCTGGT GATCCCCGGC ACACAGCCCC
2641 TGCTCATCCC GGTCGGCAGC ACTGACATGG AAGCGTCGGG GGCAGCCCCG GCCATAGTCA
2701 CGTCATCCCC CCAGTTTGCT GCAGTGCCTC ACACGTTCGT CACCACCGCC CTTCCCAAGA
2761 GCGAGAACTT CAACCCTGAG GCCCTGGTCA CCCAGGCCGC CTACCCAGCC ATGGTGCAGG
2821 CCCAGATCCA CCTGCCTGTG GTGCAGTCCG TGGCCTCCCC GGCGGCGGCT CCCCCTACGC
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2941 TGGAAGACTT AAAACAGAAG ATTTCATCCA GAGTGCAGAG ATAAGCAACG ACCTGAAGAT
3001 CGACTCCAGC ACCGTAGAGA GGATTGAAGA CAGCCATAGC CCGGGCGTGG CCGTGATACA
3061 GTTCGCCGTC GGGGAGCACC GAGCCCAGGT AACGTTAGCC AGGGTGGCAC AGGGATGGGA
3121 CACCATACCG TGATGCCATC ATCATCTCCT GGCAAGACGA ATTGCTTCTA TGAGGCAGGA
3181 TTAAGGGTTC TCGGGTACAC CTAGACCTTA GACTCGGCCT TTCCCAACTG CGTTCTCTAG
3241 AAAAAATAAG CCCCATTTCC CCGTGATCTC TGCTGTGTGT AATGAATTAA CCTCCATGCA
3301 TGGAGAGTGG GGCTAGTTAT GGAGTCCTTG AGACAATCCA GAAACTCACC ACTCTCGTTA
3361 TTTTTT
```

Patient #1 (CAG)nCACCTCAGCAGGGCTCCGGGGCTCATC; n=56.

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTCAGCAGGGCTCCGGGGCTCATC

Patient #2 (CAG)nCACCTCAGCAGGGCTCCGGGGCTCATC; n=69.

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCACCTCAGCAGGGCTCCGGGGCTCATC

Patient #3 (CAG)nCACCTCAGCAGGGCTCCGGGGCTCATC; n=47.

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCACCTCAGCAGGGCTCCGGGGCTCATC

Patient #4 (CAG)nCACCTCAGCAGGGCTCCGGGGCTCATC; n=48.

CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG
CAGCAGCAGCAGCACCTCAGCAGGGCTCCGGGGCTCATC

Patient #5 TGAG(CAG)n; n=50.

TGAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA
GCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA
GCAGCAGCAGCAGCAGCAGCAG

FIGURE 2

1 GATCCCCCCC ACCGCCAACC CCGTCACCAG TGCAGTGGCC TCGGCGCAGG

GCT-435

51 GGCCACCACT CCATCCCAGC CCTCCCAGCT GGAGGCCTAT TCCACTCTGC

Rep-2 CAG-b

101 TGGCCAACAT GGGCAGTCTG AGCCAGACGC CGGGACACAA GGCTGAGCAG

151 CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG CAGCATCAGC ATCAGCAGCA

CAG-a

201 GCAGCAGCAG CAGCAGCAGC AGCAGCAGCA GCAGCAGCAC CTCAGCAGGG

GCT-214

251 CTCCGGGGCT CATCACCCCG GGTCCCCCCC ACCAGCCCAG CAGAACCAGT

Rep-1 Pre-2

301 ACGTCCACAT TTCCAGTTCT CCGCAGAACA CCGGCCGCAC CGCCTCTCCT

351 CCGGCCATCC CCGTCCACCT CCACCCCCAC CAGACGATGA TCCCACACAC

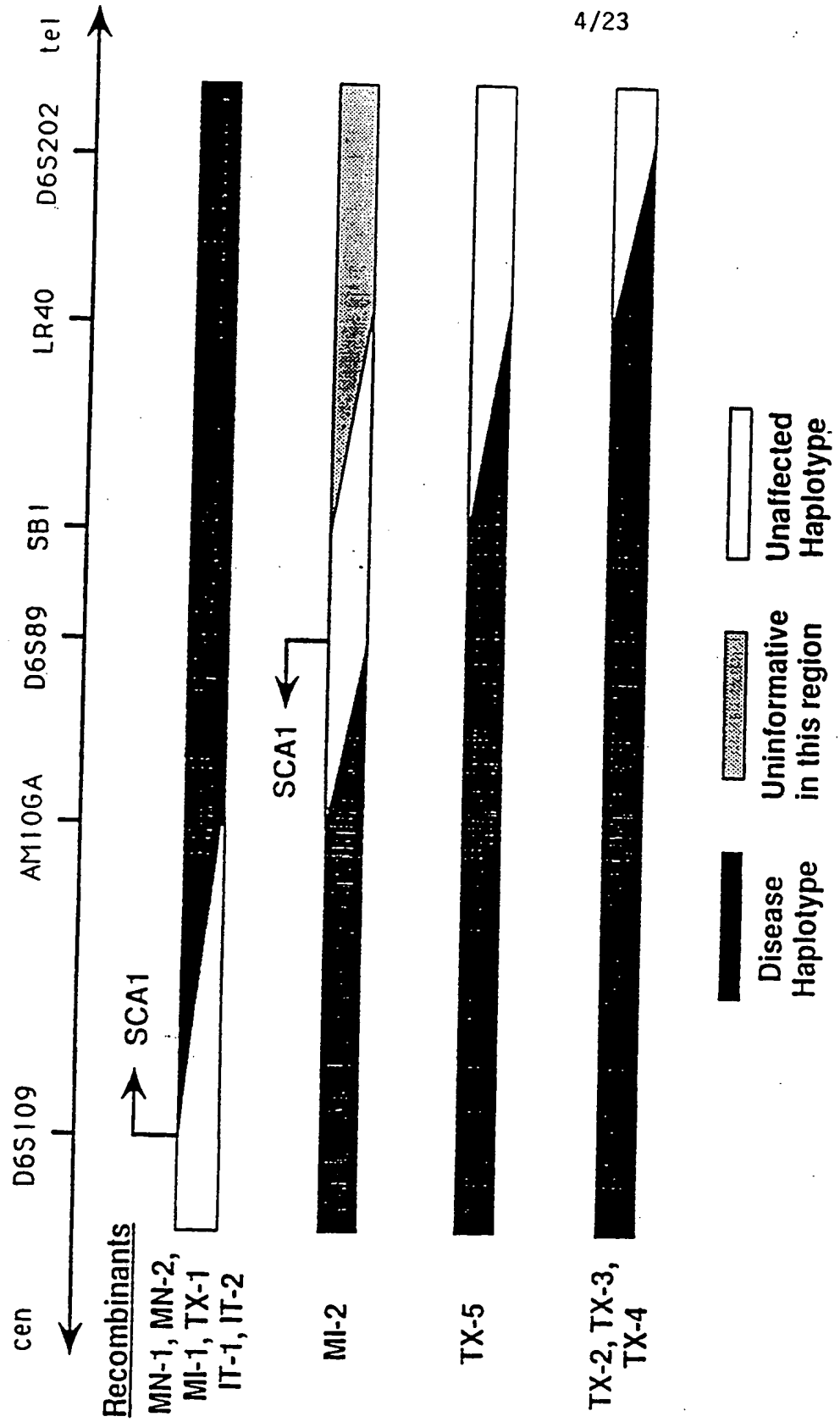
401 GCTCACCCTG GGGCCCCCCT CCCAGGTCGT CATGCAATAC GCCGACTCCG

Pre-1

451 GCAGCCACTT TGTCCCTCGG GAGGCCACCA AGAAAGCCGA GAGCAGCCGG

501 CTGCAG

Fig. 3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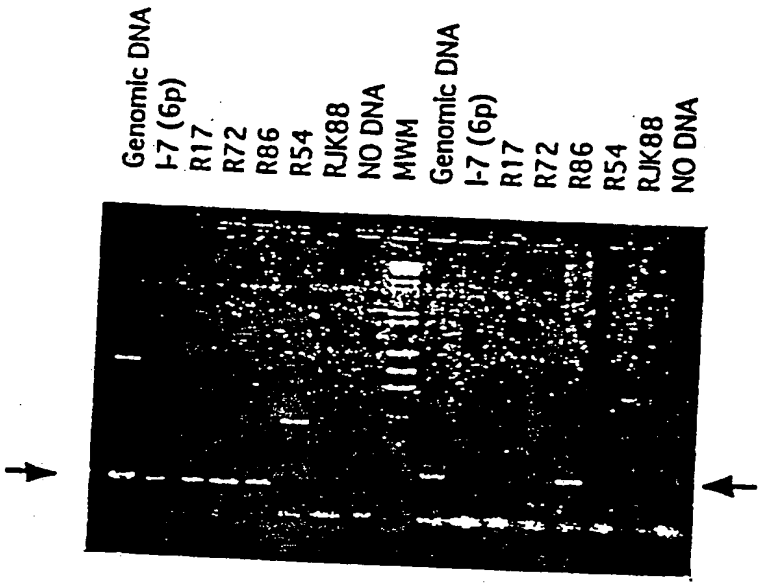
D6S109
AM10GA
D6S89
SB1
LR40
D6S202
tel
SCA1
SCA1
Recombinants
MN-1, MN-2,
MI-1, TX-1
IT-1, IT-2
MI-2
TX-5
TX-2, TX-3,
TX-4
Disease Haplotype
Uninformative in this region
Unaffected Haplotype

Fig. 4


Genomic DNA
I-7 (6p)
R17
R72
R86
R54
RJK88
NO DNA
MWM
Genomic DNA
I-7 (6p)
R17
R72
R86
R54
RJK88
NO DNA

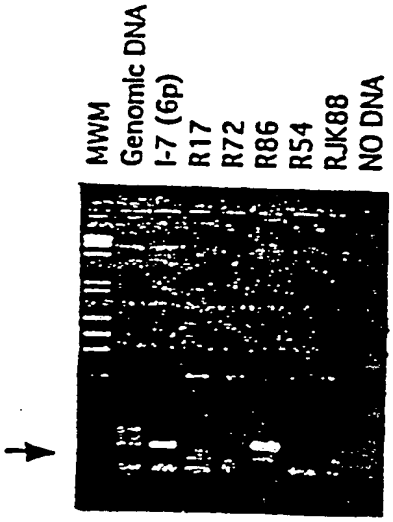
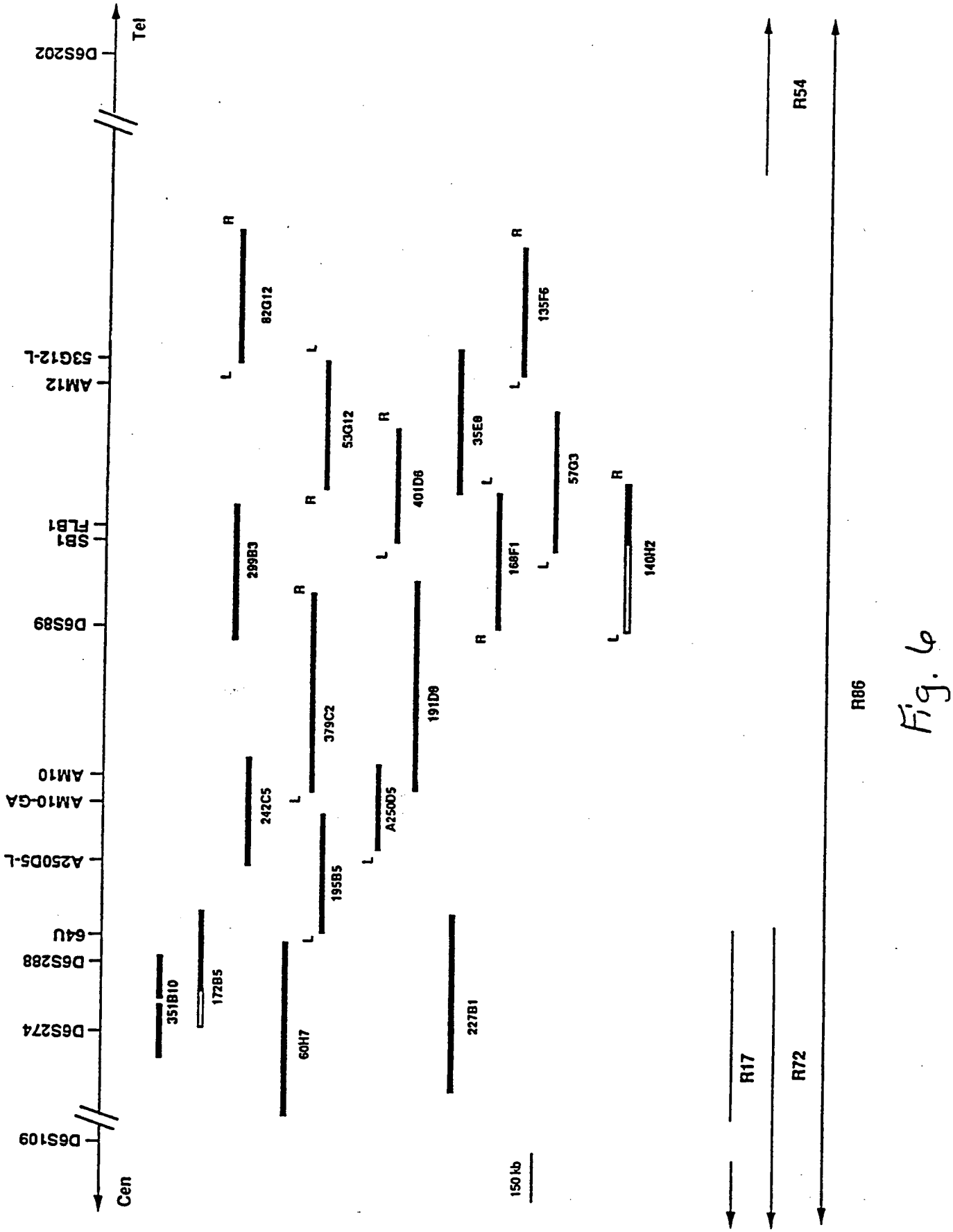

MWM
Genomic DNA
I-7 (6p)
R17
R72
R86
R54
RJK88
NO DNA

Fig. 5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AM12
SB1
FLB1
D6S89
AM10
AM10-GA
A250D5-L
64U
D6S288
D6S274
D6S109
Cen
351B10
172B5
242C5
195B5
60H7
299B3
379C2
A250D5
191D8
401D8
53G12
82G12
227B1
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168F1
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135F6
140H2
R54
R86
R17
R72
150 kb

Fig. 6

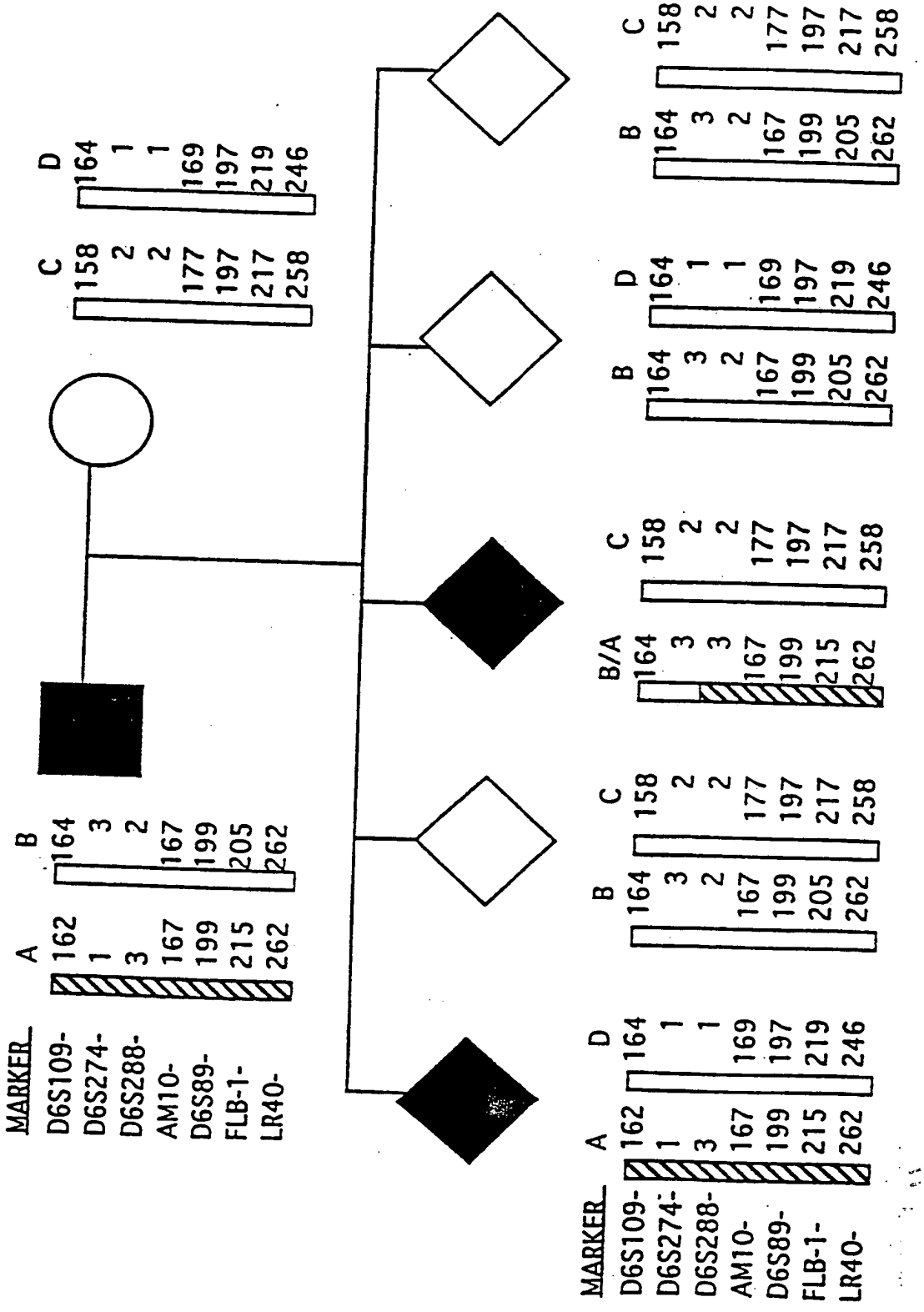
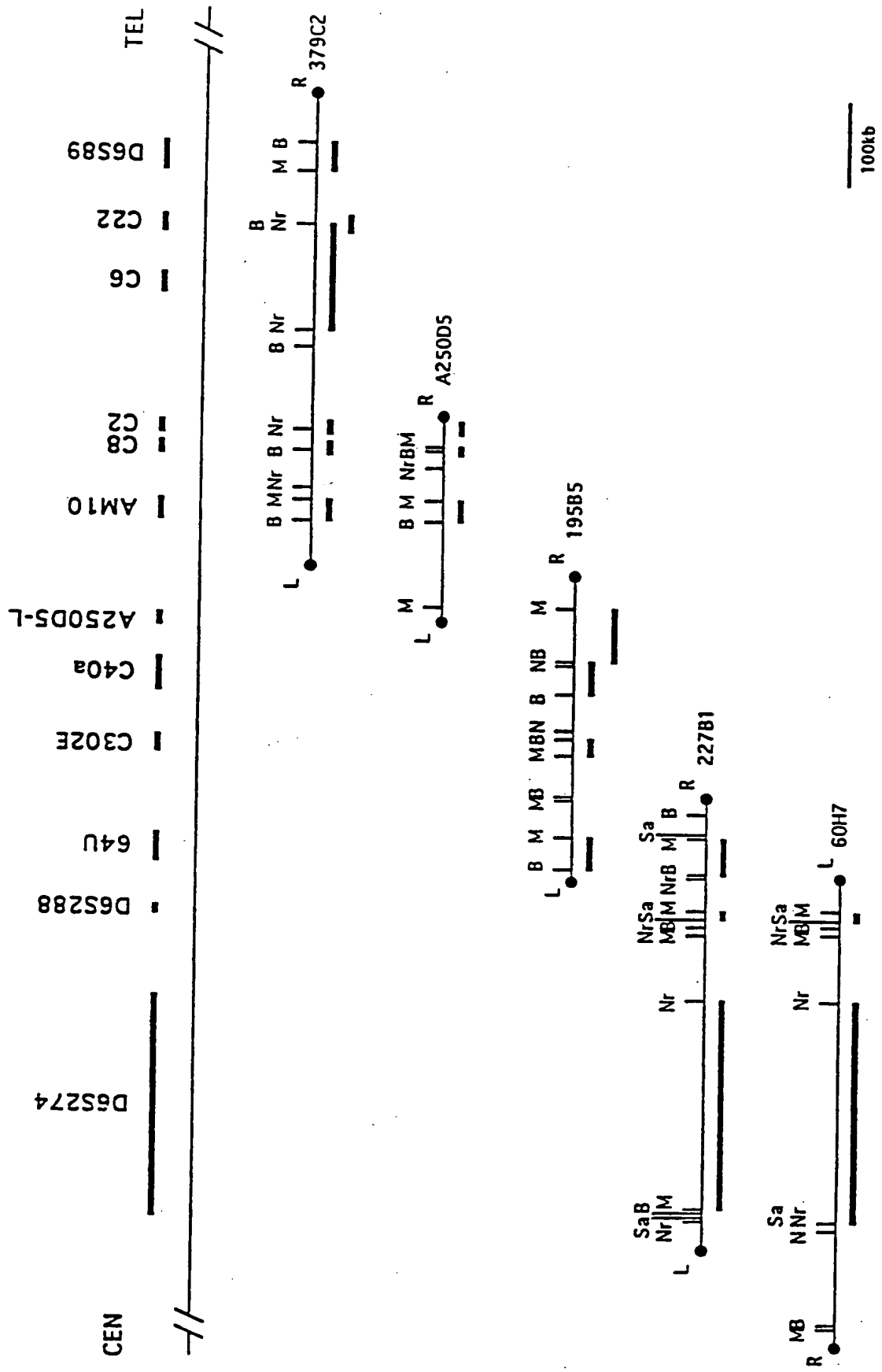

SCA1/D6S274 RECOMBINATION EVENT
MARKER
D6S109-
D6S274-
D6S288-
AM10-
D6S89-
FLB-1-
LR40-
A
162
1
3
167
199
215
262
B
164
3
2
167
199
205
262
C
158
2
2
177
197
217
258
D
164
1
1
169
197
219
246
B/A
164
3
3
167
199
215
262

Figure 7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C22
C6
C8
C2
AM10
A250D5-L
C40a
C302E
64U
D6S288
D6S274
CEN
379C2
B M B
B Nr
B Nr
B MNr B Nr
A250D5
B M NrBM
195B5
MBN B NB M
227B1
Sa
B M MB
NrSa
MB M NrB
Nr
SaB
Nr M
60H7
NrSa
MB M
Nr
Sa
N Nr
MB
100kb

Fig. 8

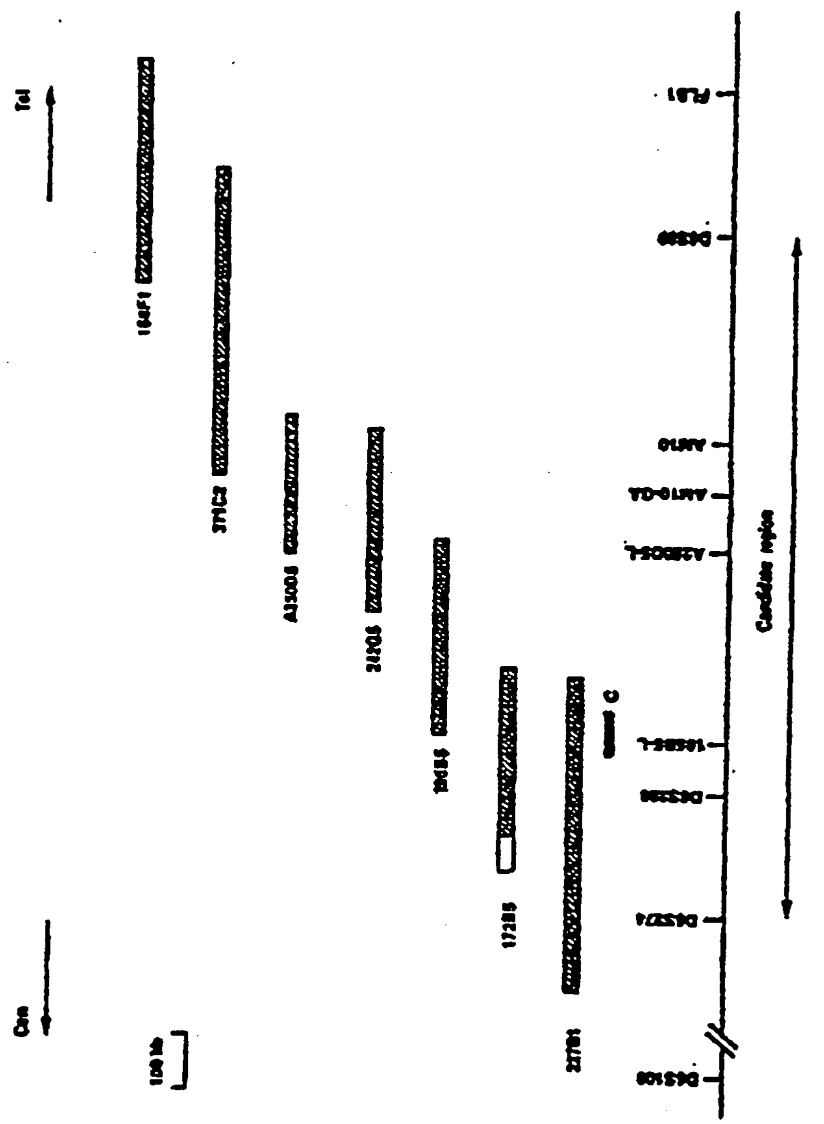
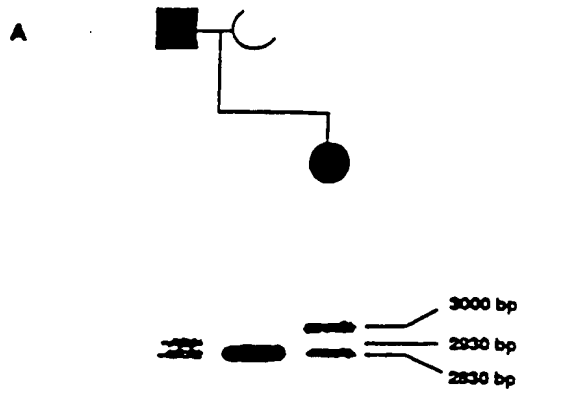
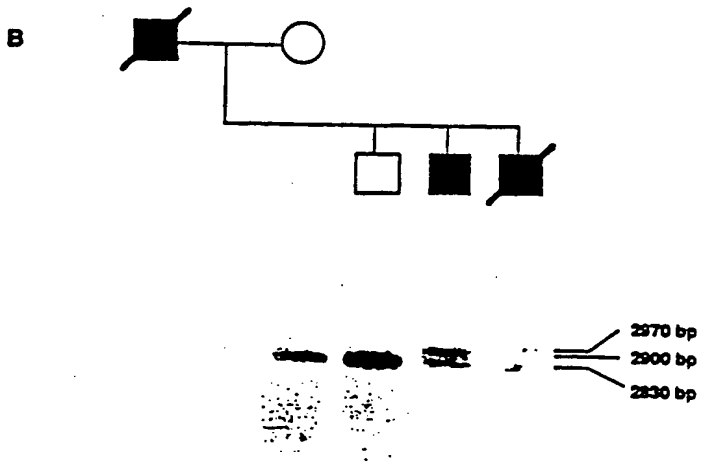


Fig. 9


A
3000 bp
2930 bp
2830 bp


B
2970 bp
2900 bp
2830 bp

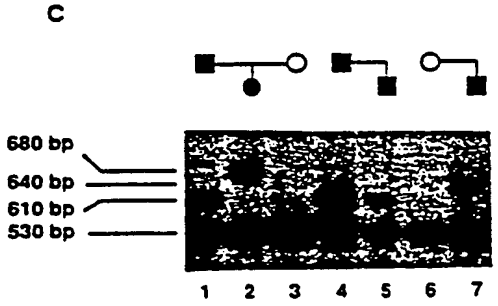

C
680 bp
640 bp
610 bp
530 bp
1 2 3 4 5 6 7

Fig. 10

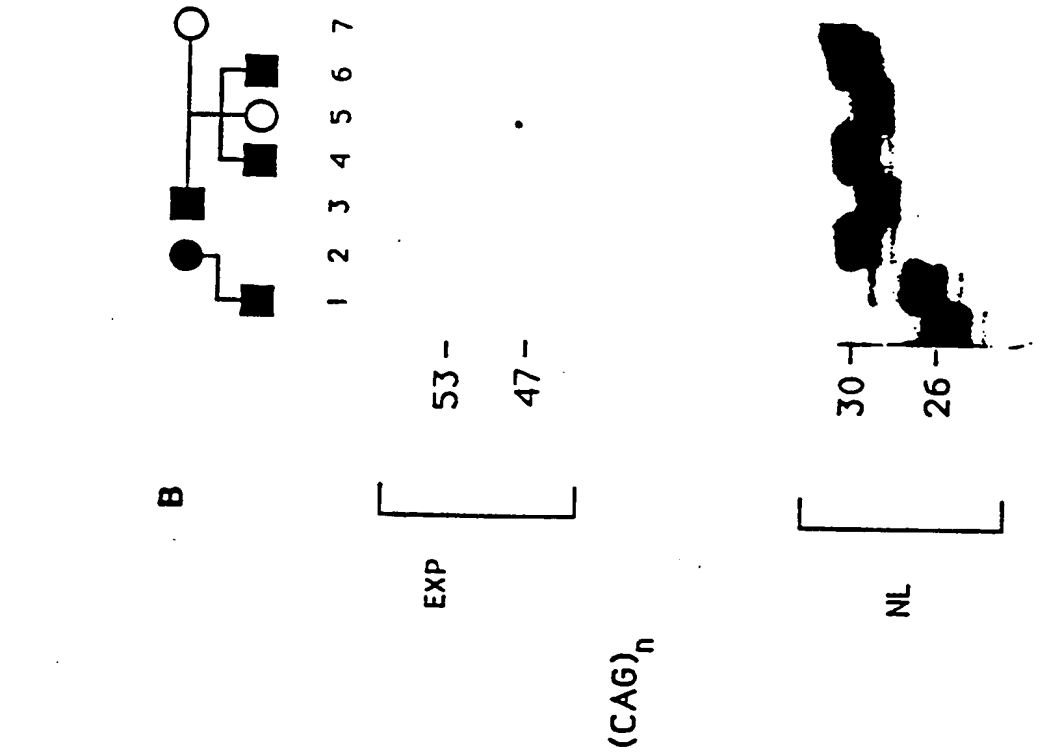
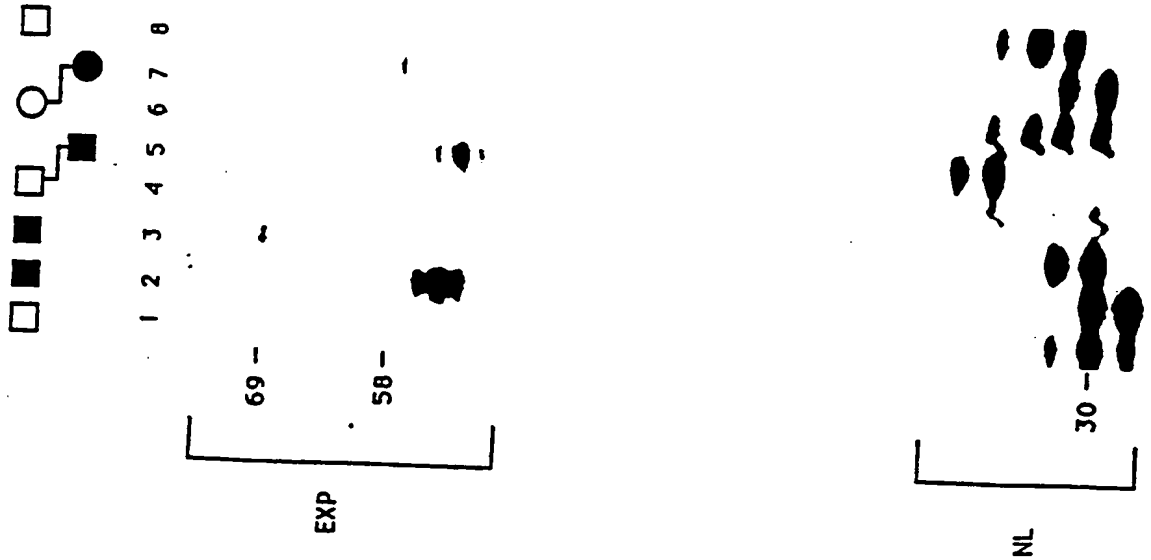


Fig. 11



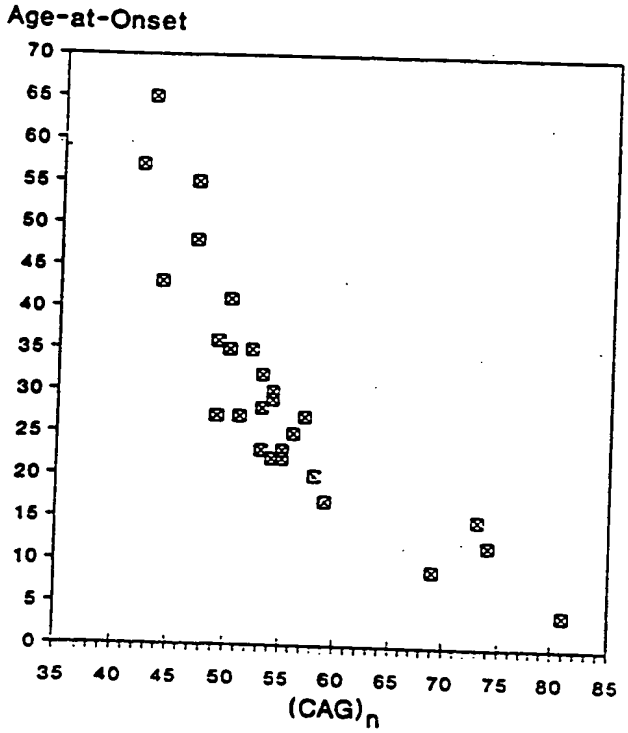

Age-at-Onset
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
35 40 45 50 55 60 65 70 75 80 85
(CAG)n

Fig. 12

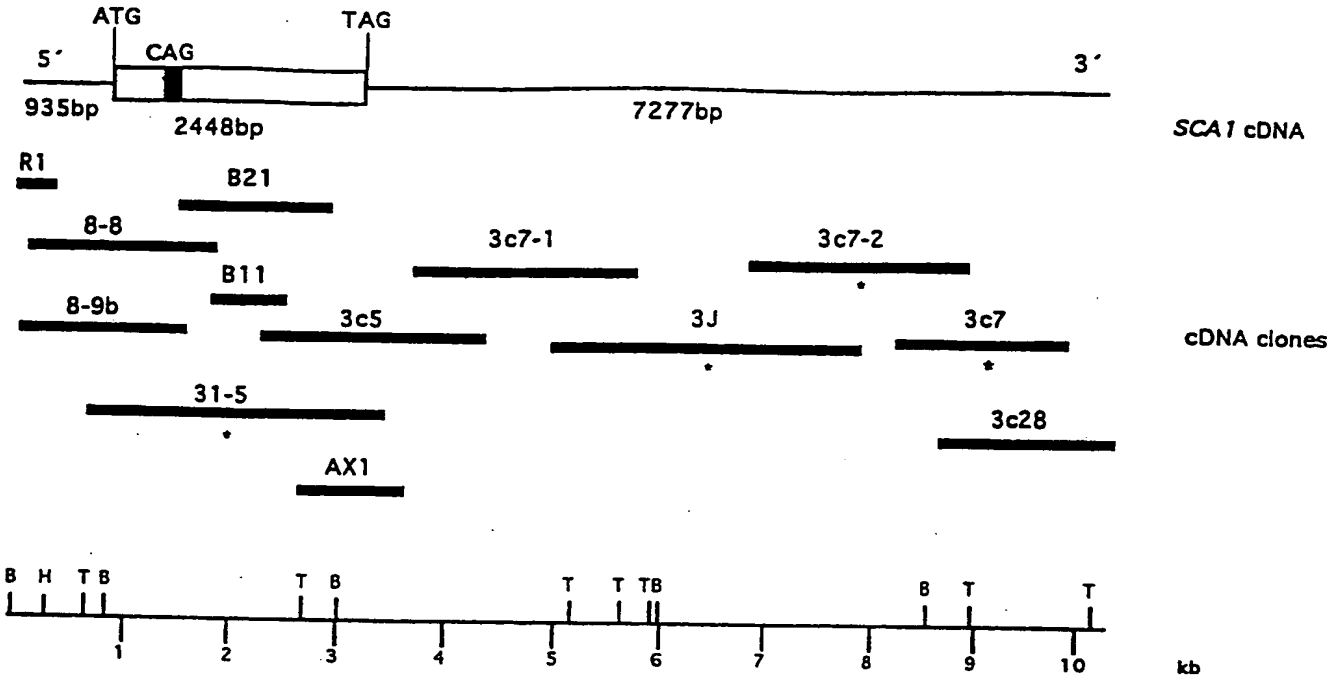

ATG
TAG
5'
CAG
3'
935bp
2448bp
7277bp
SCA1 cDNA
R1
B21
8-8
3c7-1
3c7-2
B11
8-9b
3c5
3J
3c7
cDNA clones
31-5
3c28
AX1
B H T B
T B
T
T TB
B T
T
1
2
3
4
5
6
7
8
9
10
kb

FIG. 13

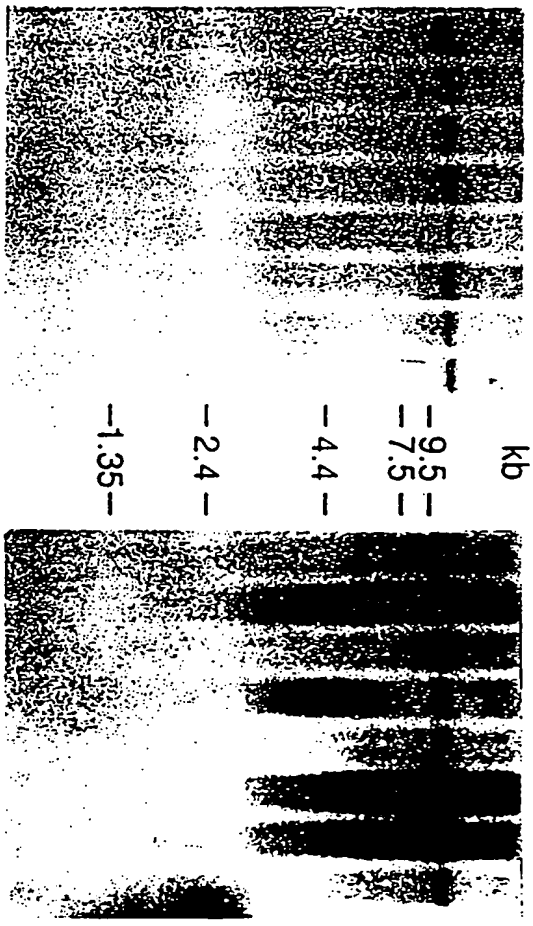

Leukocytes
Colon
Small intestine
Ovary
Testis
Prostate
Thymus
Spleen
kb
9.5
7.5
4.4
2.4
1.35
Heart
Brain
Placenta
Lung
Liver
Skel. Muscle
Kidney
Pancreas

FIG. 14

FIGURE 15

```
   1  CTACTACAGTGGCGGACGTACAGGACCTGTTTCACTGCAGGGGGATCCAAAACAAGCCCCGTGGAGCAACAGCCAGAGCAACAGCAGCTG   90
  91  CAAGACATTGTTTCTCTCCCTCTGCCCCCCCTTCCCCACGGCAACCCCAGATCCATTTACACTTTACAGTTTTACCTCACAAAAACTACTA  180
 181  CAAGCACCAAGCTCCCTGATGGAAAGGAGCATCGTGCATCAAGTCACCAGGGTGGTCCATTCAAGCTGCAGATTTGTTTGTCATCCTTGT  270
 271  ACAGCAATCTCCTCCTCCACTGCCACTACAGGGAAGTGCATCACATGTCAGCATACTGGAGCATAGTGAAAGAGTCTATTTTGAAGCTTC  360
 361  AAACTTAGTGCTGCTGCAGACCAGGAACAAGAGAGAAAGAGTGGATTTCAGCCTGCACGGATGGTCTTGAAACACAAATGGTTTTTGGTC  450
 451  TAGGCGTTTTACACTGAGATTCTCCACTGCCACCCTTTCTACTCAAGCAAAATCTTCGTGAAAAGATCTGCTGCAAGGAACTGATAGCTT  540
 541  ATGGTTCTCCATTGTGATGAAAGCACATGGTACAGTTTTCCAAAGAAATTAGACCATTTTCTTCGTGAGAAAGAAATCGACGTGCTGTTT  630
 631  TCATAGGGTATTTCTCACTTCTCTGTGAAAGGAAGAAAGAACACGCCTGAGCCCAAGAGCCCTCAGGAGCCCTCCAGAGCCTGTGGGAAG  720
 721  TCTCCATGGTGAAGTATAGGCTGAGGCTACCTGTGAACAGTACGCAGTGAATGTTCATCCAGAGCTGCTGTTGGCGGATTGTACCCACGG  810
 811  GGAGATGATTCCTCATGAAGAGCCTGGATCCCCTACAGAAATCAAATGTGACTTTCCGTTTATCAGACTAAAATCAGAGCCATCCAGACA  900
 901  GTGAAACAGTCACCGTGGAGGGGGGACGGCGAAAAATGAAATCCAACCAAGAGCGGAGCAACGAATGCCTGCCTCCCAAGAAGCGCGAGA  990
   1                                  M  K  S  N  Q  E  R  S  N  E  C  L  P  P  K  K  R  E  I   19
 991  TCCCCGCCACCAGCCGGTCCTCCGAGGAGAAGGCCCCTACCCTGCCCAGCGACAACCACCGGGGTGGAGGGCACAGCATGGCTCCCGGGCA 1080
  20    P  A  T  S  R  S  S  E  E  K  A  P  T  L  P  S  D  N  H  R  V  E  G  T  A  W  L  P  G  N   49
1081  ACCCTGGTGGCCGGGGCCACGGGGGCGGGAGGCATGGGCCGGCAGGGACCTCGGTGGAGCTTGGTTTACAACAGGGAATAGGTTTACACA 1170
  50    P  G  G  R  G  H  G  G  G  R  H  G  P  A  G  T  S  V  E  L  G  L  Q  Q  G  I  G  L  H  K   79
1171  AAGCATTGTCCACAGGGCTGGACTACTCCCCGCCCAGCGCTCCCAGGTCTGTCCCCGTGGCCACCACGCTGCCTGCCGCGTACGCCACCC 1260
  80    A  L  S  T  G  L  D  Y  S  P  P  S  A  P  R  S  V  P  V  A  T  T  L  P  A  A  Y  A  T  P  109
1261  CGCAGCCAGGGACCCCGGTGTCCCCCGTGCAGTACGCTCACCTGCCGCACACCTTCCAGTTCATTGGGTCCTCCCAATACAGTGGAACCT 1350
 110    Q  P  G  T  P  V  S  P  V  Q  Y  A  H  L  P  H  T  F  Q  F  I  G  S  S  Q  Y  S  G  T  Y  139
1351  ATGCCAGCTTCATCCCATCACAGCTGATCCCCCCAACCGCCAACCCCGTCACCAGTGCAGTGGCCTCGGCCGCAGGGGCCACCACTCCAT 1440
 140    A  S  F  I  P  S  Q  L  I  P  P  T  A  N  P  V  T  S  A  V  A  S  A  A  G  A  T  T  P  S  169
1441  CCCAGCGCTCCCAGCTGGAGGCCTATTCCACTCTGCTGGCCAACATGGGCAGTCTGAGCCAGACGCCGGGACACAAGGCTGAGCAGCAGC 1530
 170    Q  R  S  Q  L  E  A  Y  S  T  L  L  A  N  M  G  S  L  S  Q  T  P  G  H  K  A  E  Q  Q  Q  199
1531  AGCAGCAGCAGCAGCAGCAGCAGCAGCATCAGCATCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCACCTCA 1620
 200    Q  Q  Q  Q  Q  Q  Q  Q  Q  H  Q  H  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  Q  H  L  S  229
1621  GCAGGGCTCCGGGGGCTCATCACCCCGGGGGTCCCCCCCACCAGCCCAGCAGAACCAGTACGTCCACATTTCCAGTTCTCCGCAGAACACCG 1710
 230    R  A  P  G  L  I  T  P  G  S  P  P  P  A  Q  Q  N  Q  Y  V  H  I  S  S  S  P  Q  N  T  G  259
1711  GCCGCACCGCCTCTCCTCCGGCCATCCCCGTCCACCTCCACCCCCACCAGACGATGATCCCCACACACGCTCACCCTGGGGCCCCCCTCCC 1800
 260    R  T  A  S  P  P  A  I  P  V  H  L  H  P  H  Q  T  M  I  P  H  T  L  T  L  G  P  P  S  Q  289
1801  AGGTCGTCATGCAATACGCCGACTCCGGCAGCCACTTTGTCCCTCGGGAGGCCACCAAGAAAGCTGAGAGCAGCCGGCTGCAGCAGGCCA 1890
 290    V  V  M  Q  Y  A  D  S  G  S  H  F  V  P  R  E  A  T  K  K  A  E  S  S  R  L  Q  Q  A  I  319
1891  TCCAGGCCAAGGAGGTCCTGAACGGTGAGATGGAGAAGAGCCGGCGGTACGGGGCCCCGTCCTCAGCCGACCTGGGCCTGGGCAAGGCAG 1980
 320    Q  A  K  E  V  L  N  G  E  M  E  K  S  R  R  Y  G  A  P  S  S  A  D  L  G  L  G  K  A  G  349
1981  GCGGCAAGTCGGTTCCTCACCCGTACGAGTCCAGGCACGTGGTGGTCCACCCGAGCCCCTCAGACTACAGCAGTCGTGATCCTTCGGGGG 2070
 350    G  K  S  V  P  H  P  Y  E  S  R  H  V  V  V  H  P  S  P  S  D  Y  S  S  R  D  P  S  G  V  379
2071  TCCGGGCCTCTGTGATGGTCCTGCCCAACAGCAACACGCCCGCAGCTGACCTGGAGGTGCAACAGGCCACTCATCGTGAAGCCTCCCCTT 2160
 380    R  A  S  V  M  V  L  P  N  S  N  T  P  A  A  D  L  E  V  Q  Q  A  T  H  R  E  A  S  P  S  409
2161  CTACCCTCAACGACAAAAGTGGCCTGCATTTAGGGAAGCCTGGCCACCGGTCCTACGCGCTCTCACCCCACACGGTCATTCAGACCACAC 2250
 410    T  L  N  D  K  S  G  L  H  L  G  K  P  G  H  R  S  Y  A  L  S  P  H  T  V  I  Q  T  T  H  439
2251  ACAGTGCTTCAGAGCCACTCCCGGTGGGACTGCCAGCCACGGCCTTCTACGCAGGGACTCAACCCCCTGTCATCGGCTACCTGAGCGGCC 2340
 440    S  A  S  E  P  L  P  V  G  L  P  A  T  A  F  Y  A  G  T  Q  P  P  V  I  G  Y  L  S  G  Q  469
2341  AGCAGCAAGCAATCACCTACGCCGGCAGCCTGCCCCAGCACCTGGTGATCCCCGGCACACAGCCCCTGCTCATCCCGGTCGGCAGCACTG 2430
 470    Q  Q  A  I  T  Y  A  G  S  L  P  Q  H  L  V  I  P  G  T  Q  P  L  L  I  P  V  G  S  T  D  499
2431  ACATGGAAGCGTCGGGGGCAGCCCCGGCCATAGTCACGTCATCCCCCCAGTTTGCTGCAGTGCCTCACACGTTCGTCACCACCGCCCTTC 2520
 500    M  E  A  S  G  A  A  P  A  I  V  T  S  S  P  Q  F  A  A  V  P  H  T  F  V  T  T  A  L  P  529
2521  CCAAGAGCGAGAACTTCAACCCTGAGGCCCTGGTCACCCAGGCCGCCTACCCAGCCATGGTGCAGGCCCAGATCCACCTGCCTGTGGTGC 2610
 530    K  S  E  N  F  N  P  E  A  L  V  T  Q  A  A  Y  P  A  M  V  Q  A  Q  I  H  L  P  V  V  Q  559
2611  AGTCCGTGGCCTCCCCGGCGGCGGCTCCCCCTACGCTGCCTCCCTACTTCATGAAAGGCTCCATCATCCAGTTGGCCAACGGGGAGCTAA 2700
 560    S  V  A  S  P  A  A  A  P  P  T  L  P  P  Y  F  M  K  G  S  I  I  Q  L  A  N  G  E  L  K  589
2701  AGAAGGTGGAAGACTTAAAAACAGAAGATTTCATCCAGAGTGCAGAGATAAGCAACGACCTGAAGATCGACTCCAGCACCGTAGAGAGGA 2790
 590    K  V  E  D  L  K  T  E  D  F  I  Q  S  A  E  I  S  N  D  L  K  I  D  S  S  T  V  E  R  I  619
2791  TTGAAGACAGCCATAGCCCGGGCGTGGCCGTGATACAGTTCGCCGTCGGGGAGCACCGAGCCCAGGTCAGCGTTGAAGTTTTGGTAGAGT 2880
 620    E  D  S  H  S  P  G  V  A  V  I  Q  F  A  V  G  E  H  R  A  Q  V  S  V  E  V  L  V  E  Y  649
2881  ATCCTTTTTTTGTGTTTGGACAGGGCTGGTCATCCTGCTGTCCGGAGAGAACCAGCCAGCTCTTTGATTTGCCGTGTTCCAAACTCTCAG 2970
 650    P  F  F  V  F  G  Q  G  W  S  S  C  C  P  E  R  T  S  Q  L  F  D  L  P  C  S  K  L  S  V  679
2971  TTGGGGATGTCTGCATCTCGCTTACCCTCAAGAACCTGAAGAACGGCTCTGTTAAAAAGGGCCAGCCCGTGGATCCCGCCAGCGTCCTGC 3060
 680    G  D  V  C  I  S  L  T  L  K  N  L  K  N  G  S  V  K  K  G  Q  P  V  D  P  A  S  V  L  L  709
3061  TGAAGCACTCAAAGGCCGACGGCCTGGCGGGCAGCAGACACAGGTATGCCGAGCAGGAAAACGGAATCAACCAGGGGAGTGCCCAGATGC 3150
 710    K  H  S  K  A  D  G  L  A  G  S  R  H  R  Y  A  E  Q  E  N  G  I  N  Q  G  S  A  Q  M  L  739
3151  TCTCTGAGAATGGCGAACTGAAGTTTCCAGAGAAAATGGGATTGCCTGCAGCGCCCTTCCTCACCAAAATAGAACCCAGCAAGCCCGCGG 3240
 740    S  E  N  G  E  L  K  F  P  E  K  M  G  L  P  A  A  P  F  L  T  K  I  E  P  S  K  P  A  A  769
3241  CAACGAGGAAGAGGAGGTGGTCGGCGCCAGAGAGCCGCAAACTGGAGAAGTCAGAAGACGAACCACCTTTGACTCTTCCTAAGCCTTCTC 3330
 770    T  R  K  R  R  W  S  A  P  E  S  R  K  L  E  K  S  E  D  E  P  P  L  T  L  P  K  P  S  L  799
3331  TAATTCCTCAGGAGGTTAAGATTTGCATTGAAGGCCGGTCTAATGTAGGCAAGTAGAGGCAGCGTGGGGGAAAGGAAACGTGGCTCTCCC 3420
 800    I  P  Q  E  V  K  I  C  I  E  G  R  S  N  V  G  K  •                                    829
3421  TTATCATTTGTATCCAGATTACTGTACTGTAGGCTAAAATAACACAGTATTTACATGTTATCTTCTTAATTTTAGGTTTCTGTTCTAACC 3510
3511  TTGTCATTAGAGTTACAGCAGGTGTGTCGCAGGAGACTGGTGCATATGCTTTTTCCACGAGTGTCTGTCAGTGAGCGGGCGGGAGGAAGG 3600
```

Figure 15 (continued)

```
3601 GCACAGCAGGAGCGGTCAGGGCTCCAGGCATCCCCGGGGAAGAAAGGAACGGGGCTTCACAGTGCCTGCCTTCTCTAGCGGCACAGAAGC 3690
3691 AGCCGGGGGCGCTGACTCCCGCTAGTGTCAGGAGAAAAGTCCCGTGGGAAGAGTCCTGCAGGGGTGCAGGGTTGCACGCATGTGGGGGTG 3780
3781 CACAGGCGCTGTGGCGGCGAGTGAGGGTCTCTTTTTCTCTGCCTCCCTCTGCCTCACTCTCTTGCTATCGGCATGGGCCGGGGGGGTTCA 3870
3871 GAGCAGTGTCCTCCTGGGGTTCCCACGTGCAAAATCAACATCAGGAACCCAGCTTCAGGGCATCGCGGAGACGCGTCAGATGGCAGATTT 3960
3961 GGAAAGTTAACCATTTAAAAGAACATTTTTCTCTCCAACATATTTTACAATAAAAGCAACTTTTAATTGTATAGATATATATTTCCCCCT 4050
4051 ATGGGGCCTGACTGCACTGATATATATTTTTTTTAAAGAGCAACTGCCACATGCGGGATTTCATTTCTGCTTTTTACTAGTGCAGCGATG 4140
4141 TCACCAGGGTGTTGTGGTGGACAGGGAAGCCCCTGCTGTCATGGCCCCACATGGGGTAAGGGGGGTTGGGGGTGGGGGAGAGGGAGAGAG 4230
4231 CGAACACCCACGCTGGTTTCTGTGCAGTGTTAGGAAAACCAATCAGGTTATTGCATTGACTTCACTCCCAAGAGGTAGATGCAAACTGCC 4320
4321 CTTCAGTGAGAGCAACAGAAGCTCTTCACGTTGAGTTTGCGAAATCTTTTTGTCTTTGAACTCTAGTACTGTTTATAGTTCATGACTATG 4410
4411 GACAACTCGGGTGCCACTTTTTTTTTTTTCAGATTCCAGTGTGACATGAGGAATTAGATTTTGAAGATGAGCATATATTACTATCTTTAA 4500
4501 GCATTTAAAAATACTGTTCACACTTTATTACCAAGCATCTTGGTCTCTCATTCAACAAGTACTGTATCTCACTTTAAACTCTTTGGGGAA 4590
4591 AAAACAAAAACAAAAAAAACTAAGTTGCTTTCTTTTTTTCAACACTGTAACTACATTTCAGCTCTGCAGAATTGCTGAAGAGCAAGATAT 4680
4681 TGAAAGTTTCAATGTGGTTTAAAGGGATGAATGTGAATTATGAACTAGTATGTGACAATAAATGACCACCAAGTACTACCTGACGGGAGG 4770
4771 CACTTTTCACTTTGATGTCTGAGAATCAGTTCAAGGCATATGCAGAGTTGGCAGAGAAACTGAGAGAAAAGGGATGGAGAAGAGAATACT 4860
4861 CATTTTTGTCCAGTGTTTTTCTTTTTAAGATGAACTTTTAAAGAACCTTGCGATTTGCACATATTGAGTTTATAACTTGTGTGATATTCC 4950
4951 TGCAGTTTTTATCCAATAACATTGTGGGAAAGGTTTGGGGGACTGAACGAGCATAAATAAATGTAGCAAAATTTCTTTCTAACCTGCCTA 5040
5041 AACTCTAGGCCATTTTATAAGGTTATGTTCCTTTGAAAATTCATTTTGGTCTTTTTACCACATCTGTCACAAAAAGCCAGGTCTTAGCGG 5130
5131 GCTCTTAGAAACTCTGAGAATTTTCTTCAGATTCATTGAGAGAGTTTTCCATAAAGACATTTATATATGTGAGCAAGATTTTTTTTAAAC 5220
5221 AATTACTTTATTATTGTTGTTATTAATGTTATTTTCAGAATGGCTTTTTTTTTCTATTCAAAATCAAATCGAGATTTAATGTTTGGTACA 5310
5311 AACCCAGAAAGGGTATTTCATAGTTTTTAAACCTTTCATTCCCAGAGATCCGAAATATCATTTGTGGGTTTTGAATGCATCTTTAAAGTG 5400
5401 CTTTAAAAAAAAGTTTTATAAGTAGGGAGAAATTTTTAAATATTCTTACTTGGATGGCTGCAACTAAACTGAACAAATACCTGACTTTTC 5490
5491 TTTTACCCCATTGAAAATAGTACTTTCTTCGTTTCACAAATTAAAAAAAAAATCTGGTATCAACCCACATTTTGGCTGTCTAGTATTCAT 5580
5581 TTACATTTAGGGTTCACCAGGACTAATGATTTTTATAAACCGTTTTCTGGGGTGTACCAAAAACATTTGAATAGGTTTAGAATAGCTAGA 5670
5671 ATAGTTCCTTGACTTTCCTCGAATTTCATTACCCTCTCAGCATGCTTGCAGAGAGCTGGGTGGGCTCATTCTTGCAGTCATACTGCTTAT 5760
5761 TTAGTGCTGTATTTTTTAAACGTTTCTGTTCAGAGAACTTGCTTAATCTTCCATATATTCTGCTCAGGGCACTTGCAATTATTAGGTTTT 5850
5851 GTTTTTCTTTTTGTTTTTTAGCCTTTGATGGTAAGAGGAATACGGGCTGCCACATAGACTTTGTTCTCATTAATATCACTATTTACAACT 5940
5941 CATGTGGACTCAGAAAAACACACACCACCTTTTGGCTTACTTCGAGTATTGAATTGACTGGATCCACTAAACCAACACTAAGATGGGAAA 6030
6031 ACACACATGGTTTGGAGCAATAGGAACATCATCATAATTTTTGTGGTTCTATTTCAGGTATAGGAATTATAAAATAATTGGTTCTTTCTA 6120
6121 AACACTTGTCCCATTTCATTCTCTTGCTTTTTTAGCATGTGCAATACTTTCTGTGCCAATAGAGTCTGACCAGTGTGCTATATAGTTAAA 6210
6211 GCTCATTCCCTTTTGGCTTTTTCCTTGTTTGGTTGATCTTCCCCATTCTGGCCAGAGCAGGGCTGGAGGGAAGGAGCCAGGAGGGAGAGA 6300
6301 GCCTCCCACCTTTCCCCTGCTGCGGATGCTGAGTGCTGGGGCGGGGAGCCTTCAGGAGCCCCGTGCGTCTGCCGCCACGTTGCAGAAAGA 6390
6391 GCCAGCCAAGGAGACCCGGGGGAGGAACCGCAGTGTCCCCTGTCACCACACGGAATAGTGAATGTGGAGTGTGGAGAGGAAGGAGGCAGA 6480
6481 TTCATTTCTAAGACGCACTCTGGAGCCATGTAGCCTGGAGTCAACCCATTTTCCACGGTCTTTTCTGCAAGTGGGCAGGCCCCTCCTCGG 6570
6571 GGTCTGTGTCCTTGAGACTTGGAGCCCTGCCTCTGAGCCTGGACGGGAAGTGTGGCCTGTTGTGTGTGTGCGTTCTGAGCGTGTTGGCCA 6660
6661 GTGGCTGTGGAGGGGACCACCTGCCACCCACGGTCACCACTCCCTTGTGGCAGCTTTCTCTTCAAATAGGAAGAACGCACAGAGGGCAGG 6750
6751 AGCCTCCTGTTTGCAGACGTTGGCGGGCCCCGAGGCTCCCAGAGCAGCCTCTGTCACCGCTTCTGTGTAGCAAACATTAACGATGACAGG 6840
6841 GGTAGAAATTCTTCGGTGCCGTTCAGCTTACAAGGATCAGCCATGTGCCTCTGTACTATGTCCACTTTGCAATATTTACCGACAGCCGTC 6930
6931 TTTTGTTCTTTCTTTCCTGTTTTCCATTTTTAAACTAGTAACAGCAGGCCTTTTGCGTTTACAATGGAACACAATCACCAAGAAATTAGT 7020
7021 CAGGGCGAAAAGAAAAAAATAATACTATTAATAAGAAACCAACAAACAAGAACCTCTCTTTCTAGGGATTTCTAAATATATAAAATGACT 7110
7111 GTTCCTTAGAATGTTTAACTTAAGAATTATTTCAGTTTGTCTGGGCCACACTGGGGCAGAGGGGGGAGGGAGGGATACAGAGATGGATGC 7200
7201 CACTTACCTCAGATCTTTTAAAGTGGAAATCCAAATTGAATTTTCATTTGGACTTTCAGGATAATTTTCTATGTTGGTCAACTTTTCGTT 7290
7291 TTCCCTAACTCACCCAGTTTAGTTTGGGATGATTTGATTTCTGTTGTTGTTGATCCCATTTCTAACTTGGAATTGTGAGCCTCTATGTTT 7380
7381 TCTGTTAGGTGAGTGTGTTGGGTTTTTTCCCCCCACCAGGAAGTGGCAGCATCCCTCCTTCTCCCCTAAAGGGACTCTGCGGAACCTTTC 7470
7471 ACACCTCTTTCTCAGGGACGGGGCAGGTGTGTGTGTGGTACACTGACGTGTCCAGAAGCAGCACTTTGACTGCTCTGGAGTAGGGTTGTA 7560
7561 CAATTTCAAGGAATGTTTGGATTTCCTGCATCTTGTGGATTACTCCTTAGATACCGCATAGATTGCAATATAATGCTGCATGTTCAAGAT 7650
7651 GAACAGTAGCTCCTAGTAATCATAAAATCCACTCTTTGCACAGTTTGATCTTTACTGAAATATGTTGCCAAAATTTATTTTTGTTGTTGT 7740
7741 AGCTCTGGATTTTGTTTTGTTTTGTTTTTTTAAGGAAACGATTGACAATACCCTTTAACATCTGTGACTACTAAGGAAACCTATTTCTTTC 7830
7831 ATAGAGAGAAAAATCTCCAATGCTTTTGAAGACACTAATACCGTGCTATTTCAGATATGGGTGAGGAAGCAGAGCTCTCGGTACCGAAGG 7920
7921 CCGGGCTTCTTGAGCTGTGTTGGTTGTCATGGCTACTGTTTCATGAACCACAAGCAGCTCAACAGACTGGTCTGTTGCCTTCTGAAACCC 8010
8011 TTTGCACTTCAATTTGCACCAGGTGAAAACAGGGCCAGCAGACTCCATGGCCCAATTCGGTTTCTTCGGTGGTGATGTGAAAGGAGAGAA 8100
8101 TTACACTTTTTTTTTTTTTTTAAGTGGCGTGGAGGCCTTTGCTTCCACATTTGTTTTTAACCCAGAATTTCTGAAATAGAGAATTTAAGAAC 8190
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8281 GACAGTGTTGTGTTTCTGGCATAGGGAAACTCCAAACAACTTGCACACCTCTACTCCGGAGCTGAGATTTCTTTTACATAGATGACCTCG 8370
8371 CTTCAAATACGTTACCTTACTGATGATAGGATCTTTTCTTGTAGCACTATACCTTGTGGGAATTTTTTTTTAAATGTACACCTGATTTGA 8460
8461 GAAGCTGAAGAAAACAAAATTTTGAAGCACTCACTTTGAGGAGTACAGGTAATGTTTTAAAAAATTGCACAAAAGAAAAATGAATGTCGA 8550
8551 AATGATTCATTCAGTGTTTGAAAGATATGGCTCTGTTGAAACAATGAGTTTCATACTTTGTTTGTAAAAAAAAAAAGCAGAGAAGGGTTG 8640
8641 AAAGTTACATGTTTTTTTGTATATAGAAATTTGTCATGTCTAAATGATCAGATTTGTATGGTTATGGCCTGGAAGAATTACTACGTAAAA 8730
8731 GGCTCTTAAACTATACCTATGCTTATTGTTATTTTTGTTACATATAGCCCTCGTCTGAGGGAGGGGAACTCGGTATTCTGCGATTTGAGA 8820
8821 ATACTGTTCATTCCTATGCTGAAAGTACTTCTCTGAGCTCCCTTCTTAGTCTAAACTCTTAAGCCATTGCAACTTCTTTTTCTTCAGAGA 8910
8911 TGATGTTTGACATTTTCAGCACTTCCTGTTCCTATAAACCCAAAGAATATAATCTTGAACACGAAGTGTTTGTAACAAGGGATCCAGGCT 9000
9001 ACCAATCAAACAGGACTCATTATGGGGACAAAAAAAAAAAAAAATTATTTCACCTTCTTTCCCCCCACACCTCATTTAAATGGGGGGAGTA 9090
9091 AAAACATGATTTCAATGTAAATGCCTCATTTTATTTTAGTTTTATTTTGATTTTTATTTAATATAAAGAGGCCAGAATAAATACGGAGCA 9180
9181 TCTTCTCAGAATAGTATTCCTGTCCAAAAATCAAGCCGGACAGTGGAAACTGGACAGCTGTGGGGATATTAAGCACCCCCACTTACAATT 9270
9271 CTTAAATTCAGAATCTCGTCCCCTCCCTTCTCGTTGAAGGCAACTGTTCTGGTAGCTAACTTTCTCCTGTGTAATGGCGGGAGGGAACAC 9360
9361 CGGCTTCAGTTTTTCATGTCCCCATGACTTGCATACAAATGGTTCAACTGTATTAAAATTAAGTGCATTTGGCCAATAGGTAGTATCTAT 9450
9451 ACAATAACAACAATCTCTAAGAATTTCCATAACTTTTCTTATCTGAAAGGACTCAAGTCTTCCACTGCAGATACATTGGAGGCTTCACCC 9540
9541 ACGTTTTCTTTCCCTTTAGTTTGTTTGCTGTCTGGATGGCCAATGAGCCTGTCTCCTTTTCTGTGGCCAATCTGAAGGCCTTCGTTGGAA 9630
```

Figure 15 (continued)

```
 9631  GTGTTGTTCACAGTAATCCTTACCAAGATAACATACTGTCCTCCAGAATACCAAGTATTAGGTGACACTAGCTCAAGCTGTTGTCTTCAG   9720
 9721  AGCAGTTACCAAGAAGCTCGGTGCACAGGTTTTCTCTGGTTCTTACAGGAACCACCTACTCTTTCAGTTTTCTGGCCCAGGAGTGGGGTA   9810
 9811  AATCCTTTAGTTAGTGCATTTGAACTTGGTACCTGTGCATTCAGTTCTGTGAATACTGCCCTTTTTGGCGGGGTTTCCTCATCTCCCCAG   9900
 9901  CCTGAACTGCTCAACTCTAAACCCAAATTAGTGTCAGCCGAAAGGAGGTTTCAAGATAGTCCTGTCAGTATTTGTGGTGACCTTCAGATT   9990
 9991  AGACAGTCTTCATTTCCAGCCAGTGGAGTCCTGGCTCCAGAGCCATCTCTGAGACTCCGTACTACTGGATGTTTTAATATCAGATCATTA  10080
10081  CCCACCATATGCCTCCCACAGGCCAAGGGAAAACAGACACCAGAACTTGGGTTGAGGGCACTACCAGACTGACATGGCCAGTACAGAGGA  10170
10171  GAACTAGGGAAGGAATGATGTTTTGCACCTTATTGAAAAGAAAATTTTAAGTGCATACATAATAGTTAAGAGCTTTTATTGTGACAGGAG  10260
10261  AACTTTTTTCCATATGCGTGCATACTCTCTGTAATTCCAGTGTAAAATATTGTACTTGCACTAGCTTTTTTAAACAAATATTAAAAAATG  10350
10351  GAAGAATTCATATTCTATTTTCTAATCGTGGTGTGTCTATTTGTAGGATACACTCGAGTCTGTTTATTGAATTTTATGGTCCCTTTCTTT  10440
10441  GATGGTGCTTGCAGGTTTTCTAGGTAGAAATTATTTCATTATTATAATAAAACAATGTTTGATTCAAAATTTGAACAAAATTGTTTTAAA  10530
10531  TAAATTGTCTGTATACCAGTACAAGTTTATTGTTTCAGTATACTCGTACTAATAAAATAACAGTGCCAATTGCAAAAAAAAAAAAAAAAA  10620
10621  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  10660
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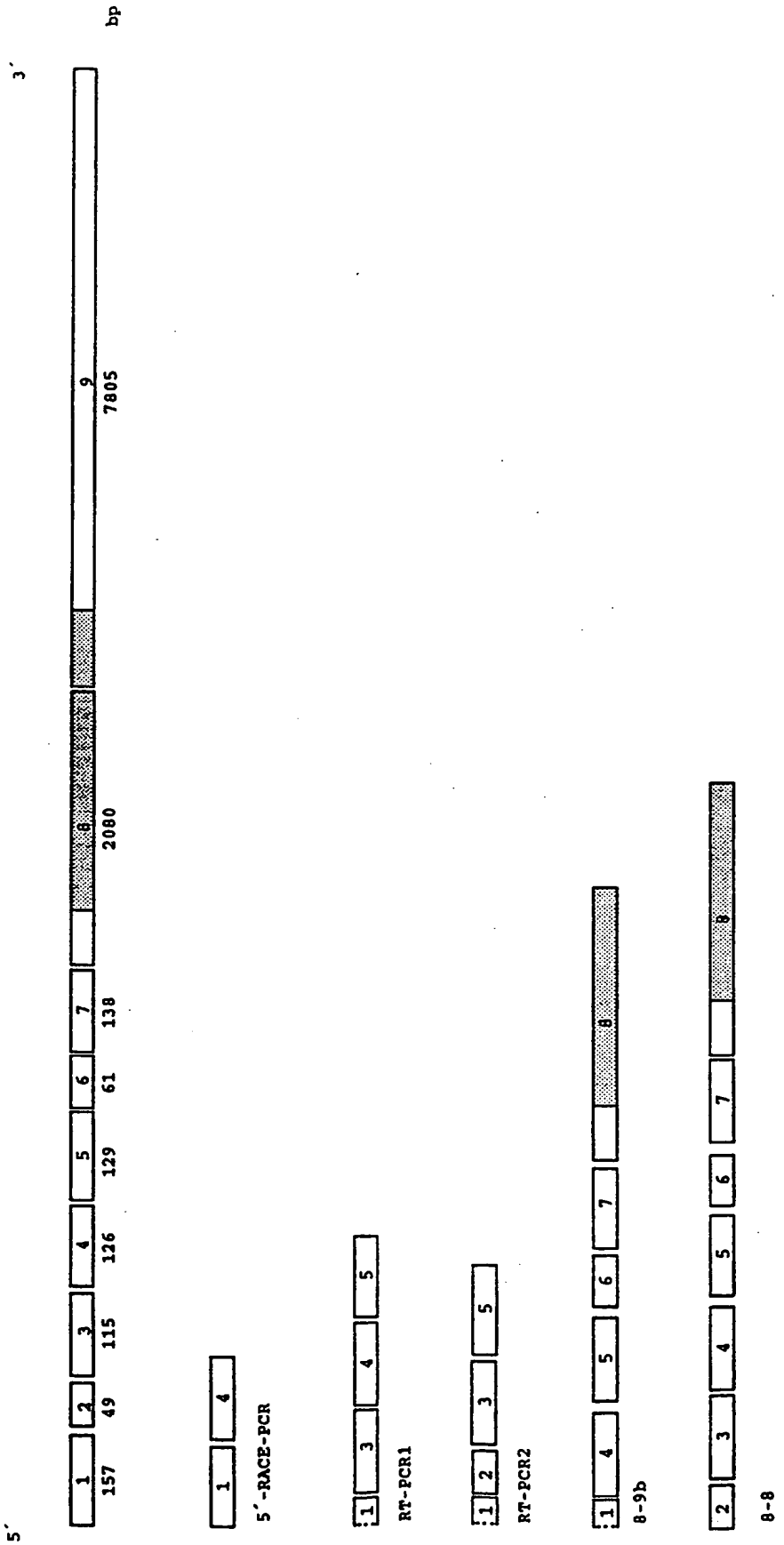

5'
3'
bp
1
2
3
4
5
6
7
8
9
157
49
115
126
129
61
138
2080
7805
5'-RACE-PCR
RT-PCR1
RT-PCR2
8-9b
8-8

FIG. 16a

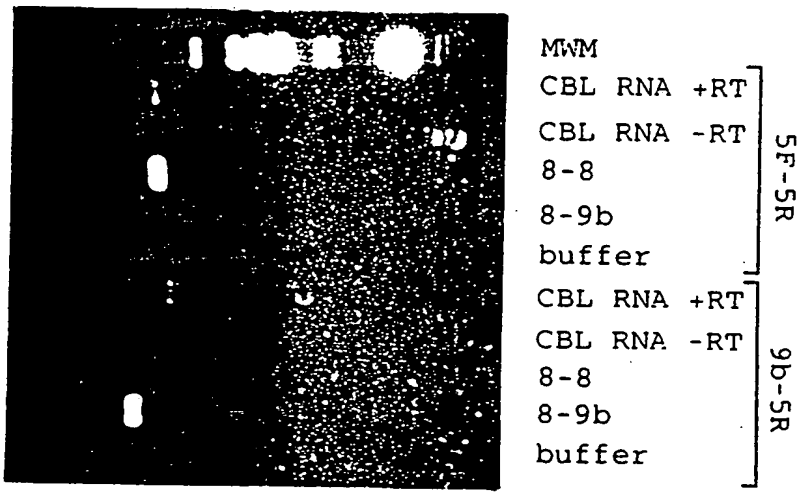
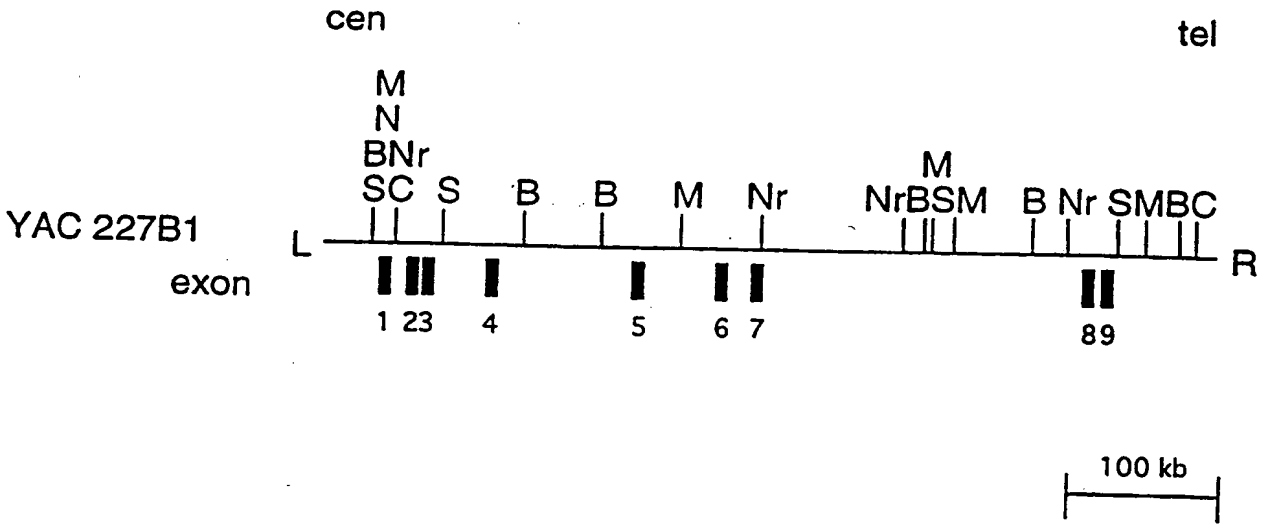

MWM
CBL RNA +RT
CBL RNA -RT
8-8
8-9b
buffer
5F-5R
CBL RNA +RT
CBL RNA -RT
8-8
8-9b
buffer
9b-5R

FIG. 16b

		Exon 1	157 TTTACA	gtaagtga
	158		206	
gtttctatgcatag	GTTTTACC	Exon 2	GGAAAG	gtatatgg
	207		321	
ctcgaccattgcag	GAGCATCG	Exon 3	TGTCAG	gtgagagt
	322		447	
ttgtttgactgcag	CATACTGG	Exon 4	TTTTTG	gtaagtca
	448		575	
ttttataattacag	GTCTAGGC	Exon 5	GTACAG	gtaaacat
	576		637	
tttttctattccag	TTTTCCAA	Exon 6	CATAGG	gtgagtga
	638		775	
tatttccatgctag	GTATTTCT	Exon 7	AATGTT	gtaagtta
	776		2855	
cttccctttcccag	CATCCAGA	Exon 8	GCCCAG	gtaacgtt
	2857			
ccctgtttccacag	GTCAGCGT	Exon 9		
YYYYYYYYYYNCAG		Consensus	AG	GTRAGT

FIG. 17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tel
M
N
BNr
SC S B B M Nr
M
NrBSM B Nr SMBC
YAC 227B1
L
R
exon
1 23 4 5 6 7 89
100 kb

FIG. 18

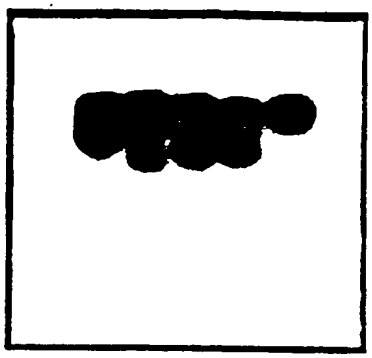

FIG. 19

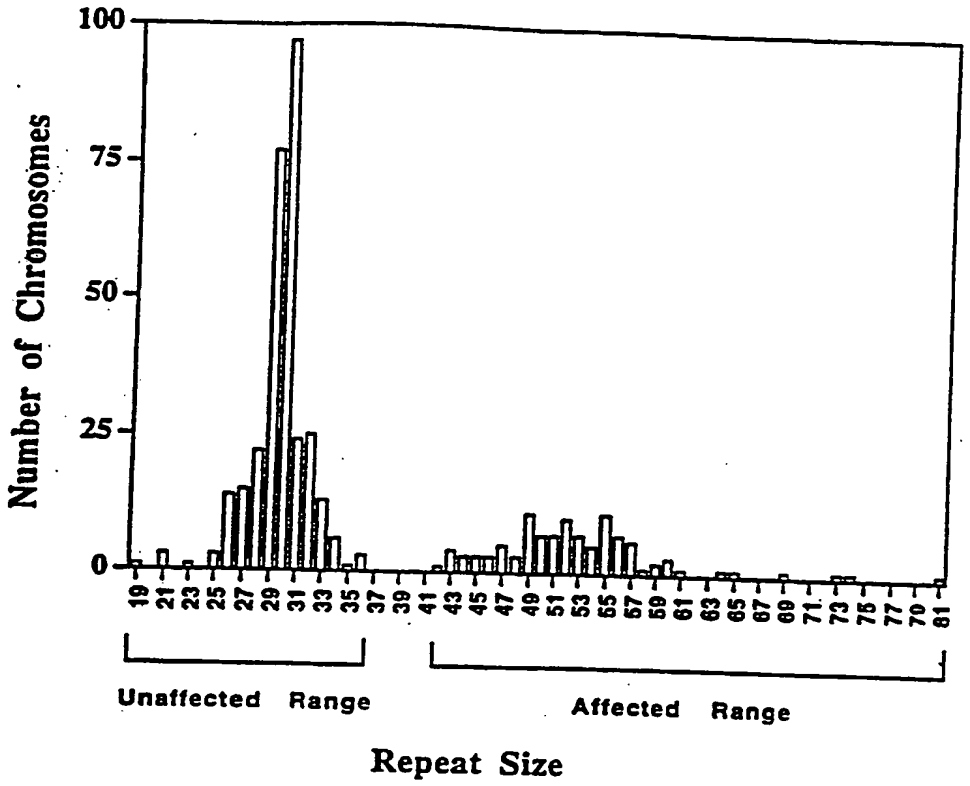
100
75
50
25
0
Number of Chromosomes
19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65 67 69 71 73 75 77 79 81
Unaffected Range
Affected Range
Repeat Size

FIG. 20